NATURE AND REGULATION OF PROTEIN PHOSPHORYLATION CHANGES DURING EGG ACTIVATION IN DROSOPHILA MELANOGASTER

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Mature oocytes are held in a developmentally-quiescent, arrested state. For development to occur, these oocytes must transition to a new cellular state that can support the processes of embryogenesis. This transition is achieved by the events of egg activation.

My studies focused on protein phosphorylation changes that take place during egg activation in Drosophila. Because there is little or no transcription at this time, egg activation is directed by maternal mRNAs and proteins regulated through post-transcriptional and post-translational mechanisms. Phosphorylation is an abundant post-translational modification with a wide array of regulatory effects. In addition, phosphorylation regulators such as CaMKII and calcineurin are required for egg activation in a variety of organisms. We hypothesize that simultaneously changing the phosphorylation states of a large number of proteins is a key contributor to the cellular changes that encompass the oocyte-to-embryo transition.

I applied two different proteomic methods, IMAC and 2D-gel electrophoresis, to identify the proteins that change in phosphorylation state between mature oocytes and unfertilized, activated eggs. This led to the identification of 311 proteins that are phospho-modulated during egg activation. I used RNAi to knock down the genes that encode some of these proteins, testing a total of 71 genes for effects on female

fertility. I identified multiple candidates for future study including, *mrityu*, which is required for progression through the early rounds of embryonic mitosis.

I also used the phosphorylation changes of two proteins identified from the proteomics experiments, Spindly and Vap-33-1, as "molecular markers" to examine how the egg activation genes *sarah*, *cortex*, and *prage* relate to the phosphorylation changes that take place at egg activation. I showed that all three genes are upstream of Spindly dephosphorylation, but only *sarah* and *cortex* are upstream of Vap-33-1 phosphorylation. These data, along with previous findings in the lab, suggest that *sarah* and *cortex* act in a common pathway.

Overall, my studies have contributed to our understanding of the roles of protein phosphorylation during egg activation. My results show that phosphorylation is an important area of study if we are to discover the proteins and pathways that regulate the oocyte-to-embryo transition.

BIOGRAPHICAL SKETCH

Amber Rose Marija Krauchunas was born and raised in New Jersey. She is the eldest daughter of Sharon Hagen and Raymond Krauchunas and has one sister, Victoria Rae Krauchunas. Her interest in genetics (and one could argue, reproduction) started early in life when her mother allowed her to breed hamsters for a seventh grade science project. This fascination with biology was cultured in her high school AP biology class, which she sometimes tried to attend twice in one day in favor of certain other classes. After high school, Amber attended Mount Holyoke College where she received a B.A. in Biological Sciences and completed requirements for minors in both mathematics and psychology. She spent her senior year doing independent research in the lab of Dr. Sharon Stranford, studying the expression of the high affinity interleukin-2 receptor in a murine model of AIDS. After graduation she spent the summer goofing off in Australia and New Jersey before moving up to Ithaca, New York to begin graduate school at Cornell University. There she spent 5 ½ years working on her Ph.D. in the lab of Dr. Mariana Wolfner, studying the phosphorylation changes that take place during egg activation in Drosophila. Her time at Cornell was also spent enjoying the natural beauty of Ithaca and the surrounding areas by hiking, fishing, and camping with friends. It was at Cornell that she also met, and began dating Jeremy Bird, who she just may marry one of these days.

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I thank the staff at Cornell's Core Life Sciences Facilities. My thesis would look a lot different without the assistance of Sheng Zhang, Bob Sherwood, and Sabine Baumgart at the Cornell Proteomics Facility. When I started at Cornell I had no knowledge of proteomics. Sheng and Bob were always incredibly friendly and more than happy to answer all of the questions that I have had over the years. Carol Bayles in the Microscopy and Imaging Facility provided training and assistance with confocal

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Finally I would like to thank lab mates, classmates, friends, and family. The Wolfner lab has been a great place to work and I have enjoyed many scientific, and non-scientific, discussions with my lab mates over the years. I especially have to thank Vanessa Horner and Kate Sackton who started the projects that drove my thesis forward. I had been told that the time spent in graduate school is an important time in one's life. Yes, you will work hard, but you will also meet people that will become good friends, maybe even life-long friends. After six years, I believe that this is true. Even if I lose touch with classmates, etc. after leaving Ithaca, the friendships I have enjoyed while here will be one of the reasons that I will always remember Cornell fondly.

Jeremy and I have experienced graduate school together. I thank him for his support and encouragement, for regular scientific discussions, for putting up with me during some of the more stressful moments (like writing this thesis), and for cooking me dinner.

And perhaps most importantly, I thank my parents. Without them I would not be here. For the obvious reason, of course, but also because of their constant support over my entire life (and because they convinced me to apply to graduate school). Not only did my parents encourage my curiosity, but they also encouraged me to seek out the answers to my questions. I will always be grateful for their love and support.

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CHAPTER ONE

INTRODUCTION[1]

Egg activation is the final transition that an oocyte goes through to become a developmentally competent egg. This transition is usually triggered by a calcium-based signal that is often, but not always, initiated by fertilization. Activation encompasses a number of changes within the egg. These include changes to the egg's membranes and outer coverings to prevent polyspermy and support the developing embryo, as well as resumption and completion of the meiotic cell cycle, mRNA poly-adenylation, translation of new proteins, and the degradation of specific maternal mRNAs and proteins. The transition from an arrested, highly differentiated cell, the oocyte, to a developmentally active, totipotent cell, the activated egg or embryo, represents a complete change in cellular state. This is accomplished by altering ion concentrations and widespread changes in both the proteome and the suite of mRNAs present in the cell. Here, we review the role of calcium and zinc in the events of egg activation, and the importance of macromolecular changes during this transition. The latter include the degradation and translation of proteins, protein post-translational regulation through phosphorylation, and the degradation of maternal mRNAs.

Introduction

At the end of oogenesis, mature oocytes are held in a developmentally-quiescent, arrested state until an appropriate trigger initiates development. In these

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specialized cells, the cell cycle is paused and maternally provided mRNAs and proteins are stored until their time to function. If development is to be successful, the oocyte must undergo one final transition from this steady state to a new cellular state that can support embryogenesis. This transition is referred to as "egg activation". Without this step of oocyte development, fertilization cannot lead to the creation of an embryo.

The events of egg activation include cortical granule exocytosis (in many taxa) and the block to polyspermy, translation of new proteins, decreases in Mitogen Activated Protein Kinase (MAPK) activity, and resumption of the cell cycle through inactivation of Maturation Promoting Factor (MPF; composed of Cdc2 kinase and cyclin B) and activation of the Anaphase Promoting Complex (APC/c). Without these changes the egg cannot limit fertilization to a single sperm, nor direct events of development such as mitosis and embryonic patterning. Egg activation as a whole process, as well as specific events such as meiotic cell cycle regulation, have been well reviewed elsewhere (Tsaadon *et al*., 2006; Horner and Wolfner; 2008b; Li *et al*., 2010; Marcello and Singson, 2010; Gadella and Evans, 2011; Liu, 2011). Here we present egg activation as it relates to a complete cellular/molecular transition, through the regulation of ion concentrations, the suite of mRNAs present within the cell, and the composition of the proteome.

Understanding how an oocyte becomes developmentally competent through egg activation is an important aspect of diagnosis and treatment of female infertility, especially as the field continues to work towards improved assisted reproductive technologies. In addition, egg activation provides a unique model system to study the

transition from a differentiated cell to a totipotent cell. In some ways, egg activation can be viewed as the converse of the change that the daughter cell of a stem cell undergoes when it differentiates, and is similar in concept to the cellular change required to form induced pluripotent cells. Upon fertilization, the highly differentiated oocyte suddenly becomes a one-cell embryo, which will give rise to all the varied cells, tissues, and organs of a complete multicellular organism. This transition represents a large and rapid change in cellular state and requires a number of molecular changes that must be both highly regulated and properly coordinated.

Ion Levels

Calcium

While the specific trigger of egg activation varies among organisms – from fertilization in vertebrates, to mechanical stimulation in some insects, to changes in pH or ionic environment for some marine invertebrates (reviewed in Horner and Wolfner, 2008b) – a rise in intracellular calcium appears to be the nearly universal signal that sets off the events of egg activation. As the regulation and role of this calcium signal has been extensively reviewed (Parrington *et al*., 2007; Horner and Wolfner, 2008b; Wakai *et al*., 2011; Nomikos *et al*., 2012), we will describe it only briefly here.

In vertebrates, fertilization leads to a rise in cytosolic Ca^{2+} in the egg which is responsible for initiating all of the events of egg activation (Horner and Wolfner, 2008b; Wakai *et al*., 2011). In mammals, this Ca^{2+} rise is mediated by a sperm-specific phospholipase C (PLCζ) (Saunders *et al*., 2002; reviewed in Wakai *et al*., 2011 and Nomikos *et al*., 2012). When a sperm enters the egg, PLCζ hydrolyzes phosphatidylinositol 4,5-bisphosphate (PIP_2) into inositol 1,4,5-triphosphate (IP_3) and

diacyl glycerol (DAG). IP_3 then binds its receptor (IP_3R1), which results in the release of Ca^{2+} from internal stores in the endoplasmic reticulum and an overall rise in cytosolic Ca^{2+} in the egg (Wakai *et al*., 2011). A recent report also showed that Ca^{2+}-influx from the external environment is necessary for complete egg activation in the mouse, suggesting greater complexity to the regulation of the Ca^{2+} signal than previously presumed (Miao *et al*., 2012).

In some organisms, Ca^{2+} levels in the egg continue to rise and fall in an oscillatory pattern following fertilization. In mammals, these oscillations last for several hours (Stricker, 1999). The importance of the oscillatory nature of the Ca^{2+} signal is still a matter of discussion, though a couple of theories have been proposed (reviewed in Wakai *et al*., 2011). Ducibella *et al*. (2002) showed that, in mouse, different egg activation events are sensitive to different numbers of calcium oscillations. For instance, a single Ca^{2+} pulse is enough to initiate cortical granule exocytosis, while resumption of the cell cycle requires at least 4 Ca^{2+} pulses (Ducibella *et al*., 2002). In addition, the number of Ca^{2+} pulses required for completion of egg activation events is greater than the number required to initiate those events; 8 Ca^{2+} pulses is not enough for polar body formation even though exit from metaphase arrest had been initiated (Ducibella *et al*., 2002). However, other evidence suggests that the Ca^{2+} oscillations are not strictly required for proper egg activation. A single large Ca^{2+} rise induced by ethanol can generate an equal number of embryos with a polar body as Ca^{2+} oscillations induced by Sr^{2+} (when observed six hours after exposure) and produces an equivalent percentage of embryos that develop full term (Rogers *et al*., 2006; Suzuki *et al*., 2010b). This has led to the proposal that

it is the overall magnitude of the Ca^{2+} increase produced by the oscillations, rather than the actual oscillatory nature of the Ca^{2+} rise, that is necessary for the events of egg activation to occur (Ducibella *et al*., 2006; Ozil *et al*., 2006).

The mechanisms of Ca^{2+} regulation at egg activation are less characterized in non-mammalian systems, though Ca^{2+} signaling itself appears to be a relatively conserved feature of the oocyte-to-embryo transition. A sperm PLC has not yet been identified outside of mammals, though a different PLC (PLCγ), along with Src-family kinases, has been implicated in the activation of the phosphoinositide pathway during fertilization in Xenopus (reviewed in Sato *et al*., 2006). In *C. elegans*, a single Ca^{2+} increase has been observed at the time of fertilization, but how this Ca^{2+} signal relates to the events of egg activation, or if it is dependent on a sperm-derived factor, is not yet known (Samuel *et al*., 2001). Supporting the idea that the Ca^{2+} rise seen in *C. elegans* plays a role analogous to that seen in other species, knocking out a *C. elegans* IP_3 receptor, *itr-1*, results in sterility, suggesting that the Ca^{2+} rise is necessary for development (Dal Santo P *et al*., 1999; Samuel *et al*., 2001). A single Ca^{2+} rise also occurs in zebrafish during egg activation (Lee *et al*., 1999). Here, the Ca^{2+} rise is dependent on the RNA-binding protein *brom bones*, which acts upstream of IP_3-mediated Ca^{2+} release (Mei *et al*., 2009). In Drosophila, *in vitro* experiments show that removal of Ca^{2+} from the buffer, through the use of the calcium chelators BAPTA or EGTA, prevents egg activation from occurring (Horner and Wolfner, 2008a). Gadolinium, which inhibits calcium influx into the cell, also prevents egg activation in this system (Horner and Wolfner, 2008a). Thus, like other species, Drosophila

requires Ca^{2+} for egg activation and some, or all, of the Ca^{2+} must come from the external environment.

A number of questions still remain regarding how well the mechanisms of Ca^{2+} regulation are conserved between invertebrates and mammals, and between species with sperm-dependent versus sperm-independent triggers of egg activation. What is clear is that for a complete cellular transition, where coordination of all the changes is key, all of the events are initiated by a single signal. An increase of cytosolic Ca^{2+} is typically the signal that triggers egg activation, after which individual events then appear to be carried out by various Ca^{2+}-binding/Ca^{2+}-dependent proteins (Figure 1.1).

Zinc

While a role for Ca^{2+} has long been established in egg activation, the regulation of Zn^{2+} levels during mammalian fertilization has gained attention in recent years. In contrast to Ca^{2+}, where increasing levels are required to activate egg activation pathways, Zn^{2+} levels decrease at egg activation. Upon fertilization or parthenogenetic activation in mouse, zinc is rapidly released from the egg in 1-5 exocytosis events termed "zinc sparks" (Kim *et al*., 2011). Zinc sparks are also observed in the two non-human primate species that have been tested (Kim *et al*., 2011). Each zinc spark is immediately preceded by a Ca^{2+} rise and none occur when Ca^{2+} rises are blocked by the calcium chelator BAPTA (Kim *et al*., 2011). This suggests that the release of Zn^{2+} may be dependent on the Ca^{2+} signal. If Zn^{2+} is artificially reduced in the mature oocyte by the zinc chelator TPEN, MAPK kinase activity decreases, cyclin B is degraded, and meiotic arrest is released (Suzuki *et al*., 2010b; Kim *et al*., 2011). These events occur in the absence of a Ca^{2+} increase in the

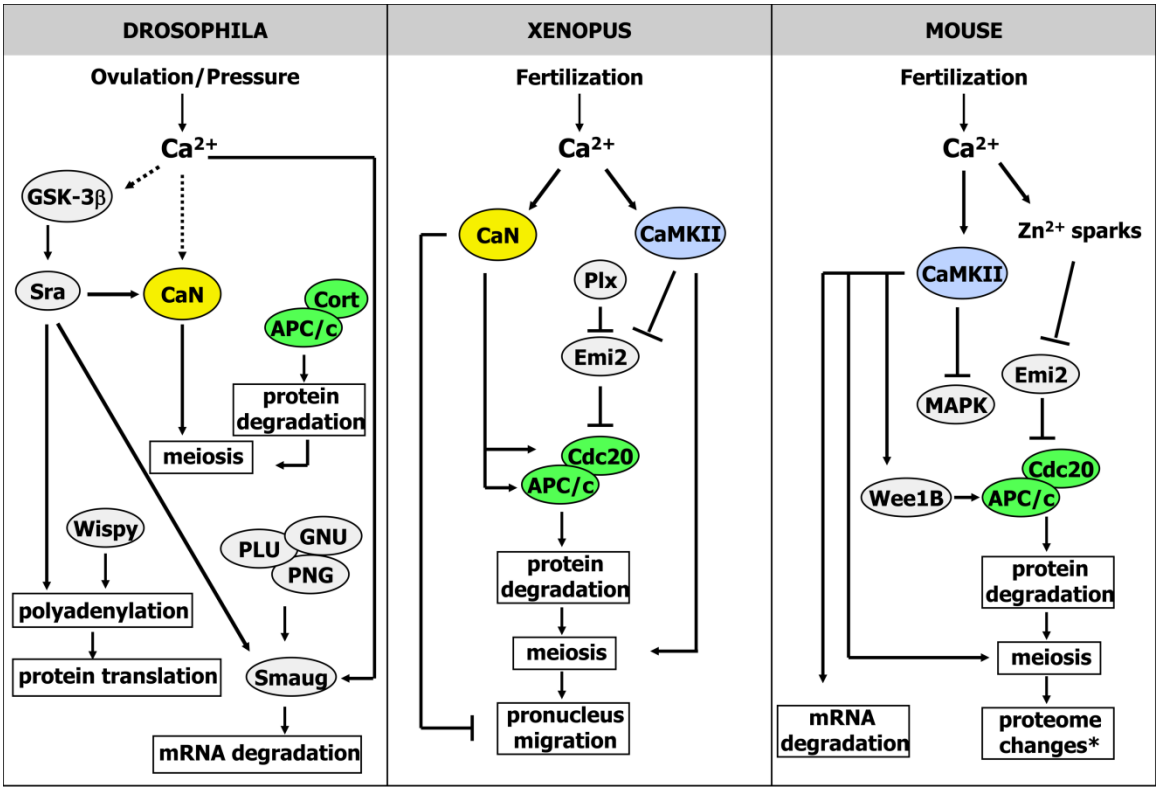

Figure 1.1 Diagram of known egg activation pathways in Drosophila, Xenopus, and mouse based on experimental results. See text for references. *Knott *et al*. (2006) and Backs *et al*. (2010) show that the protein patterns observed by 2D- and 1D-gel electrophoresis in activated mouse eggs are regulated by CaMKII and attribute these changes to maternal mRNA recruitment and protein synthesis. However, it can not be ruled out that some of the observed changes are due to post-translational modifications. As such, we have placed "proteome changes" in the pathway to encompass both of these possibilities.

oocyte and are unaffected by the addition of BAPTA, once again suggesting that the decrease of Zn^{2+} levels is downstream of the Ca^{2+} signal (Suzuki *et al*., 2010b). Zn^{2+} sequestration by TPEN appears to be sufficient to induce all the events of egg activation, as eggs fertilized with heat-inactivated sperm heads (that cannot produce a Ca^{2+} signal), and then activated with TPEN, are able to fully develop into viable mice (Suzuki *et al*., 2010b). Additionally, when zinc levels are increased with the zinc ionophore, ZnPT, mouse oocytes can no longer be parthenogenetically activated with $SrCl_2$ (Bernhardt *et al*., 2012). Thus, release of zinc is not only sufficient, but also necessary for the events of egg activation.

Precisely how Zn^{2+} release triggers exit from meiotic arrest has not yet been worked out. TPEN-induced resumption of the cell cycle is dependent on the APC/c activator Cdc20, suggesting that it requires APC/c activity (Suzuki *et al*., 2010b). However, TPEN does not induce degradation of the APC/c inhibitor Emi2 (Suzuki *et al*., 2010b). The Emi2 protein is required to maintain the metaphase II arrest in mature mouse oocytes by inhibiting the APC/c; Emi2 is typically degraded at egg activation to allow cell cycle progression (discussed below; Schmidt *et al*., 2005; Shoji *et al*., 2006). The fact that chelation of Zn^{2+} can induce resumption of the cell cycle without degradation of Emi2, along with the fact that Emi2 contains a putative zinc-binding region, has led to the model that zinc binding may directly modulate the activity of Emi2 (Bernhardt *et al*., 2012). This model is supported by the finding that a single mutation in the zinc-binding region of Emi2 completely inhibits its function (Schmidt *et al*., 2005). In addition, Zn^{2+} has been shown to affect the activity of the phosphatase Cdc25 and thus its ability to dephosphorylate Cdc2 (also known as Cdk1)

(Sun *et al*., 2007). Therefore, it is possible that decreases in Zn^{2+} levels also contribute to progression of the cell cycle by reducing the efficiency of Cdc25 and allowing Cdc2 to become phosphorylated and inhibited. Further work is required to determine if these models are correct and to identify whether additional proteins are modulated by Zn^{2+} levels in the oocyte.

Changes in Proteome Composition

Large-scale changes in the egg's proteome take place at egg activation that drastically alter the cellular properties from those of the mature oocyte. Because there is little or no transcription associated with the oocyte-to-embryo transition, changes in protein composition within the cell rely on post-transcriptional and post-translational methods of regulation. Studies have been undertaken to characterize the proteome of oocytes and/or embryos in multiple organisms including mouse, frog, sea urchin, and Drosophila and provide an overview of the dynamic changes that occur during egg activation. In the mouse, over 3500 proteins have been identified in metaphase II oocytes and approximately 2000 have been found in zygotes (Pfeiffer *et al*., 2011; Wang *et al*., 2010; reviewed in Yurttas *et al*., 2010). This discrepancy in the number of proteins identified between oocytes and zygotes is suggestive of significant degradation of maternal proteins (Wang *et al*., 2010). A functional role for this degradation is seen in sea urchins, where inhibition of the proteasome blocks proper entry into the first mitosis following fertilization (Kawahara *et al*., 2000). In agreement with this result, 2-dimensional gel analysis of unfertilized and fertilized sea urchin eggs shows a 23% decrease in the number of detectable spots 2 minutes post-fertilization (Roux *et al*., 2006). This is likely due to a combination of protein

degradation as well as post-translational modifications, as the same study showed that approximately 30% of the proteins in unfertilized eggs are phosphorylated (Roux *et al*., 2006). It is the overall combination of degradation and post-translation modification of existing maternal proteins, and new protein translation from maternal mRNAs, that transitions an oocyte into an embryo.

Protein degradation

As mentioned above, substantial protein degradation is observed upon egg activation. One important role for this degradation is the regulation of the meiotic cell cycle by the APC/c, an E3 ubiquitin-ligase that targets proteins for degradation by the 26S proteosome (reviewed in McLean *et al*., 2011). Targets of the APC/c include securin and cyclins, which hold the mature oocyte in metaphase arrest until egg activation. As there are several recent reviews on the function and regulation of the APC/c (Pensin and Orr-Weaver, 2008; Verlhac *et al*., 2010; McLean *et al*., 2011), we will only mention the key points here.

The APC/c is regulated by the adaptor protein Cdc20, which acts as an activator of the APC/c and confers substrate specificity. While the role of Cdc20 in mitosis is well studied, its role in meiosis remains more elusive (reviewed in Pesin and Orr-Weaver, 2008). However, in Drosophila, a meiosis-specific Cdc20, Cortex, has been identified that is required for cyclin degradation and completion of meiosis during egg activation (Page and Orr-Weaver, 1996; Chu *et al*., 2006; Pesin and Orr-Weaver, 2007; Swan and Schupbach, 2007). Cortex is also responsible for regulating its own degradation by the APC/c following egg activation, presumably to allow the canonical Cdc20 (Fizzy) to regulate the subsequent embryonic mitoses (Pesin and Orr-

Weaver, 2007). Continuing studies in Drosophila will provide a unique model system for understanding how Cdc20 is regulated to activate the metaphase-to-anaphase transition during egg activation.

In Xenopus and mice, a novel mechanism for regulating the APC/c during egg activation was found through the identification of Emi2/XErp1 as a necessary protein for maintaining metaphase arrest (Schmidt *et al*., 2005; Shoji *et al*., 2006). Emi2 inhibits the APC/c in metaphase-arrested oocytes/egg extracts, evidenced by the fact that removal of Emi2 results in precocious metaphase exit, while overexpression of Emi2 prevents APC/c activation upon addition of $SrCl_2$ or Ca^{2+} (Schmidt *et al*., 2005; Shoji *et al*., 2006). Upon egg activation in Xenopus, Emi2 protein is degraded by the Skp1-Cullin F-box (SCF) ubiquitin ligase complex, showing that at least one other degradation pathway besides the APC/c is active during egg activation (Schmidt *et al*., 2005). This degradation is believed to be dependent on phosphorylation by Polo-like kinase 1 (Plx1). Two Plx1 phosphorylation sites are present within the $DSGX_3S$ motif required for Emi2 degradation, and mutation of either of these sites reduces Emi2 degradation *in vivo* and Plx1 phosphorylation of Emi2 *in vitro* (Schmidt *et al*., 2005). While Emi2 is also degraded upon egg activation in the mouse, a similar mechanism of phosphorylation by Plx1, and degradation by the SCF, has not been demonstrated (Shoji *et al*., 2006). In addition, though Emi2 clearly inhibits the APC/c, the exact mechanism of this inhibition is not yet known. Emi2 has been proposed to interact with, and inhibit, both Cdc20 and the core APC/c (Shoji *et al*., 2006; Wu *et al*., 2007). Finally, it is possible that once activated, the APC/c can contribute to the inactivation of Emi2. Studies in Xenopus show that overexpression of the APC/c-cooperating E2

enzyme leads to the ubiquitylation of Emi2 and its disassociation from the APC/c (Hormanseder *et al*., 2011).

Although the APC/c clearly plays a critical role in protein degradation during egg activation, it is not known to what extent other pathways contribute to the global degradation that is observed. In Xenopus, action of the SCF is implicated by its role in Emi2 degradation, but whether it has any additional targets at this time remains to be investigated. Additionally, autophagy [a bulk degradation system of the cell (reviewed in Mizushima, 2007)] is highly induced in early mouse embryos and is required for preimplantation development (Tsukamoto *et al*., 2008a; Tsukamoto *et al*., 2008b). A full understanding of the role of protein degradation, and the ways in which individual pathways regulate unique or overlapping sets of targets, will require a more complete characterization of the proteome after egg activation and how inhibition of each pathway affects global changes in protein composition in the egg.

Protein phosphorylation

Multiple types of post-translational modification are likely to act on the proteome during egg activation. For instance, maternal sumoylation is important for eggshell patterning and mitosis during Drosophila embryogenesis (Nie *et al*., 2009). However, the main focus thus far has been on the role of phosphorylation, and that is where we will also focus in this review. In sea urchins, fertilization causes the number of phosphorylated proteins to double within 2 minutes, before returning to pre-fertilization levels 30 minutes later (Roux *et al*., 2006). In Xenopus, phosphoproteomic analysis has identified a combined total of 654 phosphoproteins in oocytes, unfertilized eggs, and embryos (McGivern *et al*., 2009). And in Drosophila,

we have identified 311 proteins that change in phosphorylation state between mature oocytes and unfertilized, activated eggs (Krauchunas *et al*., submitted).

Phosphorylation can be a major contributor to a change in cellular state since it has the ability to rapidly modulate a large number of proteins and has a wide array of regulatory affects [ie. altering protein activity (Cargnello and Roux, 2011) or localization (Jans and Hubner, 1996)]. The importance of phosphorylation during egg activation is evidenced by the dynamic phosphorylation changes that take place during the oocyte-to-embryo transition, and the finding that phosphorylation modulators such as CaMKII and calcineurin (discussed below) are critical for successful egg activation.

CaMKII

Ca^{2+}/calmodulin-dependent protein kinase II (CaMKII) is a serine/threonine protein kinase that is regulated by calcium and the calcium receptor molecule calmodulin (reviewed in Hudmon and Schulman, 2002). Because of the relationship between Ca^{2+} and CaMKII, and the importance of Ca^{2+} to initiate egg activation, CaMKII has been proposed to be one of the main effectors of the Ca^{2+} signal at egg activation. Numerous studies support this hypothesis, pointing primarily to a key role for CaMKII in the resumption of the cell cycle.

An increase in CaMKII activity occurs at the time of fertilization/egg activation that correlates with the observed increase in Ca^{2+} levels in both Xenopus and mouse. In Xenopus, where one Ca^{2+} spike occurs, CaMKII activity transiently spikes in the first 5 minutes after calcium addition (Liu and Maller, 2005; Nishiyama *et al*., 2007). In mice, the activity of CaMKII is oscillatory, once again correlating with the pattern of Ca^{2+} rises in the egg (Johnson *et al*., 1998; Tatone *et al*., 2002;

Markoulaki *et al*., 2003; Markoulaki *et al*., 2004). When Ca^{2+} oscillations are produced by fertilization, or artificially induced, CaMKII activity is seen to peak at the time of Ca^{2+} peaks followed by a return to baseline activity levels; these increases in kinase activity can be observed for up to one hour post-insemination (Markoulaki *et al*., 2003; Markoulaki *et al*., 2004). In contrast, if mouse oocytes are activated by ethanol, which produces only a single Ca^{2+} rise, CaMKII activity increases only once, showing that the oscillations of CaMKII activity are dependent on the oscillations of Ca^{2+} levels in the egg (Tatone *et al*., 2002). When CaMKII is inhibited in Xenopus or mouse oocytes, cyclin B fails to be degraded, Cdc2 remains active, and the cell cycle remains arrested in metaphase (Lorca *et al*., 1993; Morin *et al*., 1994; Johnson *et al*., 1998; Tatone *et al*., 1999; Markoulaki *et al*., 2003; Madgwick *et al*., 2005). If a constitutively active form of CaMKII is added to the oocyte, cyclin B degradation is induced, Cdc2 and MAPK levels decrease, and meiosis resumes (Lorca *et al*., 1993; Morin *et al*., 1994; Madgwick *et al*., 2005; Knott *et al*., 2006).

CaMKII exists in four different isoforms - α, β, δ, γ. In mouse oocytes, two splice forms of CaMKIIγ are predominantly expressed, while the other isoforms are present at low or undetectable levels (Backs *et al*., 2010; Chang *et al*., 2009; Suzuki *et al*., 2010a). In CaMKIIγ conditional knockout mice, or when CaMKIIγ is knocked down by an antisense morpholino, eggs remain arrested in metaphase and MAPK and Cdc2 kinase levels remain high (Backs *et al*., 2010; Chang *et al*., 2009). Injection of CaMKII cDNA rescues this phenotype. Rescue is not isoform-specific, suggesting that it is the general activity of CaMKII that is required for meiotic resumption rather than any CaMKIIγ-specific targets (Backs *et al*., 2010). In addition, characteristic

proteome changes that occur at egg activation, observed by either 2D- or 1D-gel electrophoresis, are not seen in the CaMKIIγ mutants (Backs *et al*., 2010), though they can be triggered by addition of constitutively active CaMKII (Knott *et al*., 2006). However, these proteome changes can also be induced by a Ca^{2+} signal and direct inactivation of Cdc2 kinase, even when CaMKII is not present (Backs *et al*., 2010). Thus, these observed changes in proteome composition appear to rely on resumption of the cell cycle but are only indirectly regulated by CaMKII.

Two mechanisms for how CaMKII initiates the metaphase to anaphase transition have been elucidated in Xenopus. The first comes from the finding that CaMKII is both necessary and sufficient for Emi2 degradation in Xenopus egg extracts (Rauh *et al*., 2005; Hansen *et al*., 2006). As mentioned earlier, in Xenopus, Polo-like kinase 1 (Plx1) phosphorylates Emi2 to target it for degradation (Schmidt *et al*., 2005). Removing Plx1 prevents release from the metaphase arrest, even when constitutively active CaMKII is added (Liu and Maller, 2005). CaMKII is equally required for Plx1 to induce resumption of the cell cycle (Liu and Maller, 2005). Additionally, while expression of either CaMKII or Plx1 alone cannot overcome the arrest caused by Emi2 overexpression, a combination of both kinases can (Liu and Maller, 2005). This suggests that the kinases work together to regulate Emi2 degradation and the subsequent exit from meiotic arrest. *In vitro* experiments show that Emi2 is a good substrate for CaMKII and that Emi2 interacts with, and is phosphorylated by, Plx1 in a CaMKII-dependent manner (Liu and Maller, 2005; Rauh *et al*., 2005; Hansen *et al*., 2006). Thus, the current model is that when CaMKII is activated by the Ca^{2+} rise at egg activation it phosphorylates Emi2, which primes

Emi2 for subsequent phosphorylation by Plx1, eventually leading to its degradation (Liu and Maller, 2005; Rauh *et al*., 2005; Hansen *et al*., 2006). CaMKII also promotes microtubule destabilization at the onset of anaphase (Reber *et al*., 2008). This function is independent of APC/c activation, suggesting that CaMKII works at multiple levels to regulate progression of the cell cycle at egg activation.

Whether a similar mechanism for the role of CaMKII in Emi2 degradation or microtubule destabilization exists in mammals has not yet been established. However, in mouse, the kinase Wee1B has been shown to be a CaMKII target at egg activation. Knockdown of Wee1B in oocytes results in a failure to completely activate the APC/c and high levels of MAPK in parthenogenetically activated eggs, preventing exit from metaphase (Oh *et al*., 2010). In addition, Wee1B knockdown reduces the ability of constitutively active CaMKII to induce polar body formation (Oh *et al*., 2010). *In vitro* experiments show that Wee1B can be phosphorylated by CaMKII, and that this phosphorylation increases Wee1B activity (Oh *et al*., 2010). Thus, in mouse, it has been shown that CaMKII regulates resumption of the cell cycle through phosphorylation of Wee1B, which in turn phosphorylates and inhibits Cdc2, leading to the inhibition of MPF activity and the transition from metaphase to anaphase.

Calcineurin

Calcineurin is a Ca^{2+}/calmodulin-dependent serine/threonine phosphatase composed of a catalytic A subunit (CnA) and regulatory B subunit (Rusnak and Mertz, 2000). A role for calcineurin at egg activation was first suggested by studies in Drosophila which showed that the calcineurin regulator, Sarah (Sra), is necessary for multiple aspects of egg activation (Horner *et al*., 2006; Takeo *et al*., 2006). Eggs laid

by *sra* mutant females have defects in the polyadenylation and translation of maternal mRNAs and defects in protein degradation (Horner *et al*., 2006). In addition, while meiosis briefly resumes in eggs laid by *sra* mutants, it re-arrests at anaphase I, perhaps due to the high levels of cyclin B in these eggs (Horner *et al*., 2006; Takeo *et al*., 2006).

The absence of these egg activation events in eggs laid by *sra* mutants is presumed to be due to misregulation of calcineurin when Sra is not present. This hypothesis is supported by both genetic and biochemical assays. Three calcineurin subunits are present in Drosophila oocytes and early embryos: two A subunits (Pp2B-14D and CanA-14F) and one B subunit (CanB2) (Takeo *et al*., 2006). Sra has been shown to interact with all three of these subunits, both in cell culture and *in vivo* by mass spectrometry experiments (Takeo *et al*., 2006; Takeo *et al*., 2012). In addition, genetic interaction studies showed that the phenotype caused by expression of a dominant negative CnA is rescued by Sra overexpression and enhanced in *sra* heterozygotes (Takeo *et al*., 2010). Thus, it is proposed that Sra is a positive regulator of calcineurin function during egg activation.

A direct role for calcineurin in Drosophila egg activation has been shown through germline clonal analysis of both CanB2 mutants and CnA double mutants. In both cases elimination of calcineurin function from the oocyte/embryo led to an anaphase I arrest identical to that seen in *sra* mutants (Takeo *et al*., 2010; Takeo *et al*., 2012). As the CnA double mutants can be rescued by expression of wild-type Pp2B-14D, but not by a phosphatase-dead version of the protein, the phosphatase activity of calcineurin is required for its role at egg activation in Drosophila (Takeo *et al*., 2012).

What remains to be determined is whether the defect in polyadenylation and translation seen in *sra* mutants also occurs in calcineurin mutants, or if *sra* has additional functions beyond the regulation of calcineurin. If calcineurin is indeed necessary for these events, it provides a link between the Ca^{2+} signal and maternal mRNA translation. However, the possible calcineurin target(s) regulating this event remain unidentified.

A role for calcineurin in egg activation has also been observed in Xenopus (Mochida and Hunt, 2007; Nishiyama *et al*., 2007). Addition of Ca^{2+} to Xenopus egg extracts causes a transient increase in calcineurin activity (Mochida and Hunt, 2007; Nishiyama *et al*., 2007). This transient activity occurs along the same time frame as the spike in CamKII activity that is seen upon addition of calcium, and precedes the degradation of cyclin B (Nishiyama *et al*., 2007). When calcineurin is inhibited, Cdc2 activity remains high and eggs/extracts are not able to exit metaphase II (Nishiyama *et al*., 2007). Immunodepletion of calcineurin confirms these results, showing a failure to degrade both cyclin B and securin (Nishiyama *et al*., 2007). Similar to the results in Drosophila, adding back constitutively active calcineurin reverses this phenotype but a phosphatase-dead version of the protein does not (Nishiyama *et al*., 2007). Surprisingly, constitutively active calcineurin reduces the size of sperm asters formed around demembranated sperm added to Ca^{2+}-activated Xenopus egg extracts. This is in contrast to Drosophila, where sperm asters fail to form in eggs laid by *sra* mutants (Horner *et al*., 2006). Thus, at least in Xenopus, it appears that it is important that calcineurin is activated only transiently.

The necessity of calcineurin for cyclin B degradation and metaphase II exit leads to the hypothesis that calcineurin is involved in the regulation of the APC/c. In Xenopus, it appears that calcineurin does directly regulate components of the APC/c (Mochida and Hunt, 2007). Two APC/c subunits, Apc3 (a core subunit) and Fizzy (Cdc20), are phospho-regulated during the cell cycle (Peters *et al*., 1996; Chung and Chen, 2003) and both proteins undergo dephosphorylation when calcium is added to egg extracts (Mochida and Hunt, 2007). However, the calcineurin inhibitor Cyclosporin A prevents this dephosphorylation from occurring (Mochida and Hunt, 2007). The ability of calcineurin to dephosphorylate immunopurified Apc3 and Fizzy suggests that these proteins are direct targets of the phosphatase (Mochida and Hunt, 2007). Whether calcineurin also contributes to the regulation of the APC/c through Emi2 is less clear. While Nishiyama *et al*. (2007) report that inhibiting calcineurin reduces the degradation of Emi2, Mochida and Hunt (2007) find that Emi2 dephosphorylation and degradation is unaffected in the presence of Cyclosporin A. Almost all the Ca^{2+} induced dephosphorylations that can be observed with an anti-(phospho-Ser-Pro) antibody are prevented by Cyclosporin A, suggesting that there are many more calcineurin targets that remain to be identified (Mochida and Hunt, 2007). However, how many of these dephosphorylations are direct targets, and how many are indirect effects due to misregulation of the APC/c, is unknown.

As with CamKII, calcineurin appears to be a key regulator activated by the Ca^{2+} signal that triggers egg activation. The activation of calcineurin is independent of CamKII activity, and vice versa (Nishiyama *et al*., 2007). However the transient activity of both proteins is required for metaphase II exit in Xenopus (Nishiyama *et*

al., 2007). This appears to contrast with the findings that addition of constitutively active CaMKII alone can induce resumption of the cell cycle. However, in those experiments the activity of CaMKII is not transient, the way it is under fertilization or Ca^{2+}-induced activation. When constitutively active CaMKII is inactivated 25 minutes after its addition to Xenopus egg extracts, the inactivation of Cdc2 is no longer observed unless active calcineurin is also added (Nishiyama *et al*., 2007). In addition, the results from Xenopus show that calcineurin activity and the appropriately timed inhibition of calcineurin are both required for successful egg activation and the initiation of embryogenesis. In Drosophila, the regulation of calcineurin itself is more complex than simply direct activation by Ca^{2+}, as Sra is also required for calcineurin activity. Additionally, Sra itself is regulated through phosphorylation by glycogen synthase kinase 3β (GSK-3β) (Takeo *et al*., 2012). Whether similar regulators of calcineurin are also present in Xenopus oocytes, or if GSK-3β plays a role in egg activation of other organisms remains to be tested.

At present, there is no evidence to show that calcineurin plays an analogous role in mammalian egg activation. Suzuki *et al*. (2010a) failed to detect the CnA subunit in mouse oocytes, and although ICSI-induced metaphase II exit was slowed by the addition of calcineurin inhibitors, it was not prevented. More experimentation is required to conclusively rule out any contributions that calcineurin may make to egg activation in mammals, including testing Cyclosporin A and other inhibitors when eggs are activated by Ca^{2+} (rather than ICSI), a thorough investigation into what, if any, calcineurin subunits are present in mammalian oocytes, and subsequent knockout analysis of those proteins.

Other phosphorylation regulators

While CaMKII and calcineurin have been identified as two of the most upstream regulators that transduce the Ca^{2+} signal and initiate multiple aspects of egg activation, other phosphorylation regulators have also been implicated in egg activation events and early embryogenesis. Polo-like kinase, GSK-3, Cdc25, and Wee1B have all been mentioned above. Pan Gu (PNG), is a serine/threonine kinase in Drosophila that complexes with two other proteins, Giant Nuclei (GNU) and Plutonium (PLU), to function in the oocyte-to-embryo transition (Lee *et al*., 2003). Both GNU and PLU can be phosphorylated by the PNG kinase complex *in vitro* and GNU is dephosphorylated upon egg activation, suggesting that function of the complex is itself phospho-regulated (Lee *et al*., 2003; Renault *et al*., 2003). However, other phosphorylation targets of PNG and the importance of GNU dephosphorylation remain to be determined.

In *C. elegans*, one critical phosphorylation regulator is the kinase MBK-2, which regulates maternal protein degradation during the oocyte-to-embryo transition (Pellettieri *et al*., 2003). MBK-2 is regulated by the pseudo-phosphatases EGG-3, EGG-4, and EGG-5 (Maruyama *et al*., 2007; Cheng *et al*., 2009; Parry *et al*., 2009; reviewed in Parry and Singson, 2011). Some of the targets of MBK-2 have been identified, and include major regulators of the oocyte-to-embryo transition (reviewed in Marcello and Singson, 2010). At least one role of MBK-2 phosphorylation appears to be providing a priming phosphorylation for subsequent modification by an additional kinase, as MBK-2 targets have been shown to be further phosphorylated by GSK-3 and polo kinases (Nishi and Lin, 2005; Nishi *et al*., 2008).

Other kinases that have been implicated in the oocyte-to-embryo transition of various species include protein kinase C (PKC), myosin light chain kinase (MLCK), and MAPKs. However, in many cases the roles of these kinases at egg activation are less clear than the findings on CaMKII and calcineurin. The evidence for potential functions of these kinases as they relate to the events of egg activation are reviewed elsewhere (Ducibella and Fissore, 2008).

New Protein Translation

Some of the proteins necessary for the oocyte-to-embryo transition and early embryogenesis are not present in the mature oocyte. Instead these proteins are translated from maternal mRNAs loaded into the oocyte and held in a repressed state until egg activation. Analysis of the protein classes that are required in the early embryo, as well as the function of specific proteins encoded by the maternally deposited mRNAs, will inform us of the cellular events that are critical at this time and help identify potential regulators of those processes.

One way to determine the full set of proteins that are translated in the activated egg is by analyzing the transcripts that are associated with the polysome. This analysis has been carried out in mouse oocytes and in one-cell embryos to determine which maternal mRNAs are recruited to the polysome after egg activation (Potireddy *et al*., 2006). Nearly 2000 individual mRNAs were found to have greater signals in the one-cell polysomal RNA population when compared to the oocyte polysomal RNA population (Potireddy *et al*., 2006). These RNAs were enriched for functional classes related to metabolism, transcription, and cell cycle regulation, consistent with the idea

that the proteins translated from maternal mRNAs are important for early embryonic mitosis and activation of the zygotic genome (Potireddy *et al*., 2006).

Proteomic studies comparing mouse oocytes and zygotes also provide information on protein translation at egg activation. While the overall number of proteins identified in zygotes was lower than the number of proteins in oocytes (Wang *et al*., 2010), a comparison of the identities of the proteins in each sample should reveal proteins that are present only in the zygote, indicating that they are not translated until egg activation. It will also be of interest to compare the polysomal mRNA and proteomic data sets to determine how well polysomal recruitment correlates with identification of proteins in the embryo and/or the level of saturation reached by either of these screens.

In addition to determining the identities of these proteins, another important aspect of protein translation at egg activation is understanding how maternal transcripts are regulated to ensure they are translated at the appropriate time. One mechanism by which the translation of these transcripts is regulated is through polyadenylation of the 3' untranslated regions (UTR). During oocyte maturation in Xenopus, this process is regulated by opposing polymerase and deadenylase activities (Kim and Richter, 2006). The polymerase and deadenylase are both bound to the transcript through the cytoplasmic polyadenylation element binding protein (CPEB) and the cleavage and specificity factor (CPSF), which recognize cis-elements in the 3' UTR (Kim and Richter, 2006). When maturation is signaled, CPEB is phosphorylated and the deadenylase subsequently disassociates from the transcript. As only the poly-A polymerase remains, the result is a lengthening of the poly-A tail. It is possible that

a similar mechanism works during egg activation to regulate the polyadenylation and translation that occurs at this time.

In Drosophila, the maternal protein Wispy is a cytoplasmic poly-A polymerase that is responsible for the polyadenylation of numerous targets during both oocyte maturation and egg activation (Cui *et al*., 2008; Benoit *et al*., 2008). Targets of Wispy include Bicoid and Torso, which are important for patterning the early embryo and have been previously shown to be translated upon egg activation (Driever and Nusslein-Volhard, 1988; Casanova and Struhl, 1989). In *wispy* mutants, the *bicoid* and *torso* transcripts fail to be polyadenylated in activated eggs; for *bicoid* this has been shown to result in a failure to translate the Bicoid protein (Cui *et al*., 2008; Benoit *et al*., 2008). However, lengthening of the poly-A tail alone is not sufficient for translation, as elongation of the *bicoid* poly-A tail by overexpression of a poly-A polymerase in the oocyte does not result in Bicoid protein expression in oocytes (Juge *et al*., 2002). Thus, it appears that protein translation at egg activation not only requires the lengthening of maternal mRNA poly-A tails, but either an additional activational pathway or the repression of an inhibitory pathway that is present in the mature oocyte.

Wispy is not necessary for all protein translation during egg activation as translation of another protein, Smaug, is unaffected in *wispy* mutants (Cui *et al*., 2008; Benoit *et al*., 2008). Smaug is important for degradation of maternal transcripts after egg activation (discussed below) (Tadros *et al*., 2007). To ensure maternal transcripts are protected from Smaug-mediated degradation prior to egg activation, Smaug protein is not present in the mature oocyte (Dahanukar *et al*., 1999; Smibert *et al*.,

1999). Instead, maternal *smaug* mRNA is actively translated at egg activation; a process dependent on both *pan gu* and *sra* (Tadros *et al*., 2007; Cui *et al*., 2008). When *pan gu* is mutated *smaug* mRNA polyadenylation is also reduced (Tadros *et al*., 2007). Overexpression of a poly-A polymerase can rescue the poly-A tail phenotype of the *pan gu* mutant, however Smaug protein is still not translated, suggesting that Pan Gu works at multiple levels to control the translation of Smaug (Tadros *et al*., 2007). Pan Gu is also required for the translation of cyclin B in the early Drosophila embryo (Vardy and Orr-Weaver, 2007). In the case of cyclin B, loss of Pumilio (a translational repressor) can restore cyclin B translation in a *pan gu* mutant background, suggesting that at least one additional function of Pan Gu is to inhibit Pumilio (Vardy and Orr-Weaver, 2007). However, Smaug translation is not restored in *pumilio*; *pan gu* double mutants, indicating that additional mechanisms of Pan Gu translational regulation still remain to be discovered (Tadros *et al*., 2007).

mRNA Degradation

The mature oocyte is stocked full of maternal mRNAs that were transcribed and loaded into the oocyte during oogenesis. In Drosophila, it is estimated that up to 55% of the genes in the genome are represented in maternal transcripts in the oocyte (Tadros *et al*., 2007). For the transition to a totipotent cell (the embryo), the transcriptional profile of the differentiated cell (the oocyte) must be removed. Thus, while some of the maternal mRNAs are translated in the early embryo, another key facet of egg activation is the targeted degradation of other maternal mRNAs.

Maternal transcript degradation in the early embryo has been observed in a number of species including mouse, zebrafish, and Drosophila (Hamatani *et al*., 2004;

Mathavan *et al*., 2005; Tadros *et al*., 2007; Thomsen *et al*., 2010). In Drosophila, egg activation is both necessary and sufficient for this maternal mRNA degradation, at least for the specific transcripts examined so far (Bashirullah *et al*., 1999; Tadros *et al*., 2003). While the mechanistic connection between the Ca^{2+} signal and maternal mRNA degradation remains elusive, studies in Drosophila provide some insight into the regulation of this process. Here, the maternal protein Smaug is required for two-thirds of the transcript degradation that is observed upon egg activation (Tadros *et al*., 2007). Studies of two known Smaug targets, *nanos* and *Hsp83*, show that Smaug acts by binding to cis-elements, known as Smaug Recognition Elements (SREs), in maternal transcripts and recruiting the CCR4/POP2/NOT-deadenylase complex (Smibert *et al*., 1996; Semotok *et al*., 2005; Semotok *et al*., 2008). This leads to the deadenylation of these transcripts and their subsequent degradation. In addition, it has been proposed that Smaug interacts with the piRNA pathway to promote the deadenylation and degradation of a subset of maternal transcripts (Rouget *et al*., 2010).

Once the zygotic genome is activated, a second wave of maternal transcript degradation is activated which relies on zygotic, as well as maternal, products (Bashirullah *et al*., 1999). We will not discuss this second round of degradation and instead refer you to two comprehensive reviews on the maternal and zygotic regulation of mRNA degradation during egg activation and the maternal-to-zygotic transition by Tadros and Lipshitz (2005; 2009) and the role of microRNAs in maternal transcript degradation by Giraldez (2010). These authors also discuss the possible biological consequences of maternal transcript degradation, presenting potential roles

in producing localized expression of RNA and proteins or acting as a permissive phenomenon to avoid interfering with zygotic transcripts.

Summary

Egg activation represents a cellular transition from a highly differentiated oocyte into a totipotent embryo. There is a single initiating signal at the start of this transition, which ensures that all of the downstream signals and events are properly coordinated within the cell This is especially important considering each event is regulated at multiple levels. During egg activation, a rise in cytosolic Ca^{2+} independently activates the effector proteins CaMKII and calcineurin, and at least one other pathway, to initiate this cellular transition. [At least one other pathway must be involved as cortical granule exocytosis occurs in the absence of both calcineurin and CaMKII, and CaMKII only partially contributes to the membrane block to polyspermy (Mochida and Hunt, 2007; Nishiyama *et al*., 2007; Chang *et al*., 2009; Backs *et al*., 2010; Gardner *et al*., 2007).] These effectors then have the potential to amplify the initial signal through phosphorylation of multiple target proteins, activating multiple pathways within the cell (Figure 1.1).

Because the oocyte-to-embryo transition represents a complete change in cellular state, it requires global changes in the composition of both RNAs and proteins within the cell. Indeed, large-scale degradation of maternal mRNAs and proteins, translation of new proteins not present in the oocyte, and protein phosphorylation changes occur during egg activation. To fully understand how the embryo differs from the oocyte will require a complete identification of the molecules that are removed (degraded) and the molecules that take their place. In addition, a consistent

trend in the literature is the proposal that finding the targets of the phosphorylation regulators CamKII and calcineurin will be critical to improving our understanding of how these molecules effect the cellular changes that encompass egg activation. Identifying the proteins that are phosphorylated/dephosphorylated during egg activation will provide new proteins and pathways to explore in connecting Ca^{2+} to all of the egg activation events. Xenopus is the only species in which CaMKII and calcineurin are both known to be critical for egg activation. At present there is no evidence of a role for calcineurin in mouse egg activation, or for CaMKII in Drosophila egg activation. However, the possibility that roles for both pathways are conserved has not been definitively excluded and remains an important area for future studies.

Our current knowledge of egg activation shows that overlapping regulatory mechanisms ensure these cellular changes are tightly controlled. For example, Emi2 has been shown to be regulated by phosphorylation and subsequent degradation, ubiquitination, and Zn^{2+} levels. Following that, Emi2 is only one regulator of the APC/c, which is also regulated by the activator protein Cdc20 and phosphorylation of other core subunits. All of this works together so that the APC/c can activate the metaphase-to-anaphase transition, while at the same time activation of Wee1B kinase phosphorylates and inhibits Cdc2 (which is necessary for the metaphase arrest). Presumably, in an activated egg, all of these regulatory mechanisms are coordinated by proteins downstream of the Ca^{2+} signal to ensure the efficient transition from meiotic arrest to embryonic mitosis. Understanding how these regulatory mechanisms inter-relate, or if they simply represent redundancy within the system, will be critical

to our success in understanding this type of cellular transition, as well manipulating it therapeutically.

Dissertation Outline

My studies encompassed two aspects of the protein phosphorylation changes that take place during egg activation in Drosophila: the identification of proteins that change in phosphorylation state, and how these phosphorylation changes are regulated. I used proteomic, molecular, and genetic techniques to examine the phosphoproteome, observe specific phosphorylation changes, and test new candidates for roles in egg activation.

In Chapter Two, I present the results from two proteomic methods aimed at identifying the proteins that are phospho-modulated during egg activation. Proteins from mature oocytes and unfertilized activated eggs were separated by 2-dimensional gel electrophoresis and stained to visualize phosphorylated proteins. Spots that changed in changed in intensity for the phosphorylation stain, but not total protein abundance, were selected for identification by mass spectrometry. I also used a peptide-based approach in which proteins were first trypsin-digested and then samples were enriched for phospho-peptides by Immobilized Metal Affinity Chromatography (IMAC). Peptide sequences were analyzed by mass spectrometry and the corresponding proteins were determined. Combining results from the two methods produced 311 protein identifications. Chapter Two also contains the results of knocking down the genes encoding 18 of these proteins to test their roles in female fertility, including the identification of *mrityu* as a maternal effect gene required for the first few embryonic mitosis.

In Chapter Three, I tested the relationship between three egg activation genes *sarah*, *cortex*, and *prage* and the phosphorylation changes of Spindly and Vap-33-1 that occur at egg activation. I show that all three genes are required for Spindly dephosphorylation, but only *sarah* and *cortex* are required for Vap-33-1 phosphorylation. I also show that *sarah* is required for the degradation of Cort at egg activation, but *prage* is not. Combined with data from a previous graduate student, Kate Sackton, these results suggest that *sarah* and *cortex* act in the same pathway. *Prage* regulates only a subset of the proteins that are regulated by *sarah* and *cortex*. Therefore I hypothesize that *prage* acts either downstream, or in parallel to the *sarah/cortex* pathway.

Chapter Four contains the summaries of two different screens that sought to identify new genes that are required for egg activation or early embryogenesis. The first is a continuation of the RNAi experiments presented in Chapter Two, testing the phospho-proteomic candidates for roles in female fertility. The second is a suppressor screen designed to identify genes that interact with calcineurin in the female germline. In Chapter Four, I also present preliminary observations of Vap-33-1 localization in the developing oocyte and early embryos. I show that *mrityu* is not required for the phosphorylation changes of YA, GNU, Spindly, or Vap-33-1, consistent with a role for *mrityu* immediately after egg activation. Finally I discuss my progress in creating a null allele of *mrityu* through imprecise P-element excision mutagenesis.

Appendix A contains the results from my rotation project investigating the role of *nplp3* in the female post-mating response.

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CHAPTER TWO

PROTEIN PHOSPHORYLATION CHANGES REVEAL NEW CANDIDATES IN THE REGULATION OF EGG ACTIVATION AND EARLY EMBRYOGENESIS IN D. MELANOGASTER[2]

2.1 Introduction

For development to initiate, a number of events must take place to convert a mature oocyte to an egg capable of supporting embryogenesis. These include resumption and completion of the meiotic cell cycle, changes to the egg's outer coverings, degradation of certain maternal mRNAs, and the poly-adenylation and translation of others. These events are collectively termed "egg activation" [reviewed in (Ducibella and Fissore, 2008; Horner and Wolfner, 2008)]. In many organisms, egg activation is triggered by fertilization and a subsequent rise in intracellular Ca^{2+} (Eisen et al., 1984; Gilkey et al., 1978; Swann and Yu, 2008). However, in *Drosophila* and other insects examined, egg activation occurs independent of fertilization (Doane, 1960). In these organisms, egg activation is triggered instead by passage of the oocyte through the reproductive tract (Heifetz et al., 2001; Went and Krause, 1974), though the importance of calcium remains conserved (Horner and Wolfner, 2008a). Thus, *Drosophila,* with its ease of genetic manipulation, is a valuable model for studying the

[2] This chapter has been been accepted to Developmental Biology as Krauchunas, A.R., Horner, V.L., and Wolfner, M.F., "Protein phosphorylation changes reveal new candidates in the regulation of egg activation and early embryogenesis in D. melanogaster". The first set of 2D gels were performed by Vanessa Horner. Figure 2.1 and Table 2.1 are from Vanessa Horner's dissertation (2008) and are acknowledged in the legends.

conserved features of egg activation without interference from otherwise concurrent post-fertilization events.

However, traditional screening methods for recessive maternal-effect mutations have identified only a small number of factors necessary for egg activation in model systems such as *Drosophila* and *C. elegans* [reviewed in (Horner and Wolfner, 2008; Singson et al., 2008)]. One explanation for this modest success to date is that factors important for the oocyte-to-embryo transition may play additional essential roles at later stages of development, causing lethality and preventing their detection in a maternal-effect screen. Additionally, large-scale genetic screens to identify mutations affecting egg activation are difficult to carry out and sterile mutants can be difficult to maintain in stock. We take a different approach by identifying proteins that are post-translationally regulated during egg activation. We hypothesize that these regulated proteins will include new factors important for egg activation and early embryogenesis.

As little to no transcription occurs during egg activation, maternally provided mRNAs and proteins must be sufficient to regulate all of the events that take place during and immediately after this transition. Indeed, considerable evidence suggests that maternal proteins direct the earliest events in egg activation (McKnight and Miller, 1976; Newport and Kirschner, 1982; Zalokar, 1976). In *Drosophila*, meiosis can complete without the synthesis of any new proteins (Page and Orr-Weaver, 1997). In addition, proteomic studies of sea urchin eggs showed that in the first 2 minutes after fertilization the number of protein spots detectable by 2D gel electrophoresis decreases by 23%, suggesting that translation of new proteins is a relatively "late"

event of egg activation (Horner and Wolfner, 2008; Roux et al., 2006). Characterization of the *C. elegans* oocyte proteome and transcriptome shows that the oocyte proteome appears to be biased towards factors likely to act immediately upon fertilization while the oocyte transcriptome is biased towards factors that are likely to act later in embryogenesis (Chik et al., 2011). These findings point to maternal proteins playing key roles in the initial events of egg activation. As these proteins are present in the oocyte prior to egg activation, they must also be highly regulated so that the correct subsets of proteins are active before, during, and after egg activation is triggered.

One possible mechanism for rapid regulation of many proteins is modulation of their phosphorylation state. Protein phosphorylation/dephosphorylation occurs quickly, consistent with the time frame of early activation events. Phosphorylation can also cause a large array of regulatory effects, such as altering a protein's activity, localization, and/or association with other proteins [for examples see (Cargnello and Roux, 2011; Poon and Jans, 2005)]. Additionally, a single kinase or phosphatase can act on many substrates [for example, MAPK (Cargnello and Roux, 2011)], allowing the rapid transmission of an upstream signal to multiple downstream targets. Thus, regulation through phosphorylation can coordinate multiple events that are responsive to a single trigger. At the same time, the substrate specificity of these enzymes also allows for individual pathways to be regulated independently.

The importance of protein phosphorylation changes during egg activation is underlined by the findings that one critical regulator of this process is a kinase (CaMKII), while another is a phosphatase (calcineurin). Ca^{2+}/calmodulin-protein

kinase II (CaMKII) is required for meiotic cell cycle progression at egg activation in both mammals and amphibians (Backs et al., 2010; Chang et al., 2009; Hansen et al., 2006; Liu and Maller, 2005; Markoulaki et al., 2004; Rauh et al., 2005; Tatone et al., 1999). In *Xenopus,* the phosphatase calcineurin must be both activated and subsequently inactivated for exit from metaphase II and proper migration of male and female pronuclei (Mochida and Hunt, 2007; Nishiyama et al., 2007). In *Drosophila,* calcineurin and its regulator *sarah* are required for multiple aspects of activation, including completion of meiosis and translation of new proteins (Horner et al., 2006; Takeo et al., 2010; Takeo et al., 2006).

While these protein phosphorylation regulators are clearly important, few of the proteins being phospho-modified during egg activation have been identified. In *Drosophila,* two proteins necessary for early embryogenesis are known to be dephosphorylated during egg activation – Giant Nuclei [GNU; (Lee et al., 2003; Renault et al., 2003; Tadros et al., 2007)] and Young Arrest [YA; (Lin and Wolfner, 1991; Sackton et al., 2009; Yu et al., 2002; Yu et al., 1999)]. In vertebrates, phosphorylation of the Emi2 protein at egg activation triggers its degradation, which is necessary for the resumption of meiosis (Liu and Maller, 2005; Rauh et al., 2005). It is also known that MAPKs are dephosphorylated upon egg activation in vertebrates, as well as in *Drosophila*, leading to a decrease in MAPK activity in the egg (Fan and Sun, 2004; Sackton et al., 2007). We hypothesize that, in addition to these examples, many more maternal proteins change in phosphorylation state at this time to rapidly permit the large change in cellular state that occurs at egg activation. Consistent with this hypothesis, proteomic methods have detected dynamic changes in

phosphorylation at the time of fertilization/egg activation in sea urchins (Roux et al., 2006; Roux et al., 2008).

Here, we report the identification of proteins whose phosphorylation state changes upon egg activation in the genetic model system *Drosophila melanogaster*. Using multiple proteomic methods we identify 311 proteins that are phospho-regulated at the time of egg activation. These proteins fall into a number of functional classes that are biologically relevant to this developmental time, including calcium binding and regulation, proteolysis, and protein translation. Through a pilot RNAi screen we show that one of these proteins, Mrityu, is important for early embryogenesis. We also find that the majority of these phospho-regulated proteins are conserved from *Drosophila* to vertebrates. These methods should be broadly applicable to other systems as an efficient way to identify important molecules that act in egg activation and early embryogenesis. As these proteins are not only present, but also regulated, during this critical transition, they provide a targeted set of candidates to test in future studies for roles in the regulatory cascades and downstream events of egg activation.

2.2 Materials and Methods

Fly culture: *Drosophila melanogaster* stocks were raised on yeast-glucose-agar medium at 23 ± 2^{o}C in a 12-h L:12-h D photoperiod. All experiments were carried out with females from the P2 strain of Oregon R (ORP2) (Allis et al., 1977).

Collection of mature oocytes and laid eggs: Stage 14 mature oocytes were obtained from 3-5 day old wild-type virgin females that had been reared on heavily-yeasted

food. Dissection was performed in Isolation Buffer, a hypertonic solution that does not activate eggs (Page and Orr-Weaver, 1997). Oocytes were dissected in one hour blocks of time, then flash-frozen in liquid nitrogen and stored at –80°C until use.

Newly eclosed virgin females were aged on yeasted vials for 3-5 days and then mated to spermless males (Boswell and Mahowald, 1985) (produced by crossing *tud*^{1} *bw sp* ♀ x Canton-S ♂) to obtain unfertilized, but activated eggs. For all experiments with activated laid eggs, mated females were allowed to deposit eggs on petri plates containing grape juice-agar for 0-30 minute periods. Eggs were washed off the plates in Egg Wash (Karr and Alberts, 1986), frozen in liquid nitrogen and stored at –80°C until use. Eggs collected for Western blotting were dechorionated in a 50% bleach solution for 2 minutes before being flash frozen.

Immunoblotting: Samples of 50-100 mature oocytes or activated eggs were homogenized in Extraction Buffer (10 mM Tris pH 7.5; 20 mM NaF, 2 mM EGTA, 10 mM DTT, 400 nM okadaic acid, and 2% SDS), followed by the addition of an equal amount of SDS sample buffer. Extracts were electrophoresed on polyacrylamide SDS gels containing 0-25 μM Phos-tag (Wako Pure Chemical Industries, Ltd., Richmond, VA) and subjected to Western blotting analysis as previously described (Kinoshita et al., 2009; Kinoshita et al., 2006; Sackton et al., 2007). Primary antibodies, kind gifts of H. Bellen, R. Vale, J. Raff, F.R. Jackson, and T. Hays were used at the following dilutions in 1% milk: guinea pig anti-Vap-33-1, 1:10,000 (Pennetta et al., 2002); rabbit anti-Spindly, 1:1000 (Griffis et al., 2007); rabbit anti-Spd-2, 1:500 (Dix and Raff, 2007); rabbit anti-lark, 1:5000 (Newby and Jackson, 1996); mouse anti-DLIC, 1:3000 (Mische et al., 2008). HRP-conjugated secondary antibodies were used at a 1:2000

dilution and visualized with the ECL Plus Western Blotting Detection system (GE Healthcare, Piscataway, NJ).

Immunostaining and microscopy: Embryos and ovaries were fixed in methanol/heptane and stained with DAPI, or as described in (Horner et al., 2006). For DAPI staining, fixed embryos were incubated in PBS containing 1 μg/ml DAPI for 5 minutes, followed by five 15 minute washes in PBST. Rat anti-sperm tail antibody (kind gift of T. Karr) was used at a dilution of 1:800 (Karr, 1991; T. Karr, ASU, personal communication). Mouse anti-tubulin antibody was used at 1:400 (Sigma, St. Louis, MO, catalog#T5168) and Alexa secondary antibodies were used at 1:200 (Invitrogen, Grand Island, NY). RNaseA (Roche, Indianapolis, IN) was added at a final concentration of 5 μg/ml and propidium iodide (Molecular Probes, Eugene, OR) was used at a final concentration of 10 μg/ml. Images were collected using a Zeiss Axioskop compound microscope or a Leica TCS SP2 confocal microscope as described in (Horner et al., 2006).

mrityu RNAi flies: A fly line with an RNAi construct to the gene CG1216 (*mrityu*) was obtained from the Transgenic RNAi Resource Project at Harvard Medical School (line GL00033). Females from this line were crossed to nanos-GAL4 males (Bloomington stock 4937) to specifically knockdown *mrityu* in the female germline (referred to as RNAi females). Control females were nanos-GAL4/TM3 siblings.

2D-gel electrophoresis: Oocytes and embryos, stored at –80^{o}C until use, were consolidated into batches of approximately 1500 mature oocytes or unfertilized laid eggs and homogenized in 50 μl protease inhibiting buffer (Monsma and Wolfner, 1988) containing 1 mM Na-orthovanadate, 10 mM β-glycerophosphate, and 20 mM

NaF as phosphatase inhibitors (PPIHB). Buffer exchange was performed three times for extracted proteins against 500 µl lysis buffer (7 M urea/2 M thio urea, 4% CHAPS) using a 10 kDa cut-off membrane filter (Millipore, Billerica, MA). Protein concentrations were determined by the Bradford assay using BSA as a standard, and approximately 100 µg of oocyte or egg protein was used for each of the 2-D gel analyses. The first-dimensional separation was performed by immobilized pH gradient isoelectric focusing (13cm IPG, nonlinear pH 3-10 strips; GE Healthcare). Isoelectric focusing was conducted using Multiphor II (GE Healthcare) according to the manufacturer's instructions after 12 h in-gel rehydration. For electrophoresis in the second dimension, the strips were then transferred and apposed to 12.5% SDS-polyacryalmide vertical gels that were cast in-house at 14 x 16 cm using a Hoefer SE600 gel cast apparatus and run using a Hoefer vertical gel running system (Hoefer, Inc., Holliston, MA). Peppermint Stick molecular weight markers (Molecular Probes) were applied to each gel at a concentration of 0.25 µg/protein. Gels were first run to observe the extent of phosphorylation changes occurring during egg activation. A second set of gels was run for protein identification from specific spots that changed in phosphorylation. Both oocyte and egg samples were run either in triplicate (first set) or duplicate (second set).

Two-dimensional electrophoresis was performed by the Cornell Proteomics Facility in the Life Sciences Core Laboratories Center.

Gel staining and imaging: Gels were fixed in 40% methanol/10% acetic acid and stored overnight in 10% methanol/7% acetic acid. Each gel was then incubated in Pro-Q Diamond Phosphorylation Stain (Molecular Probes) for the detection of

phosphoproteins. Following de-staining, the gels were scanned using a Typhoon 9400 laser scanner (GE Healthcare) with 532 nm excitation and 560 nm long pass emission filters. The gels were further post-stained with SYPRO-Ruby (Molecular Probes) or Colloidal Coomassie Blue (Invitrogen) for detection of all protein spots and once again scanned with the Typhoon 9400 with 488 nm excitation and 610 nm band pass 30 emission filters.

Gel staining and imaging were performed by the Cornell Proteomics Facility in the Life Sciences Core Laboratories Center.

Gel image analysis: Images from Pro-Q Diamond and Colloidal Coomassie Blue (CCB) or SYPRO-Ruby stains were analyzed using the Image Master 2D Platinum software (GE Healthcare).

For the first set of gels, the replicate Pro-Q Diamond and CCB images for each sample type (mature oocyte or activated egg) were combined into a single Pro-Q or CCB synthetic gel image, containing the average volume of each spot. Once values were obtained for each spot, Pro-Q spot volumes were normalized by dividing each spot by its corresponding CCB spot volume. Two known phosphorylated proteins, ovalbumin and beta-casein, present in the PeppermintStick protein marker were used to set a Pro-Q/CCB ratio minimum which we used to determine which spots were phosphorylated. The lowest Pro-Q/CCB ratio obtained for these control proteins was 0.9. Therefore, a protein spot was considered phosphorylated if the Pro-Q/CCB ratio was greater than or equal to 0.9. Due to this normalization process, only the Pro-Q spots with a matching CCB spot could be analyzed for potential phosphorylation. The

final normalized values were used in comparison between samples, with a 2-fold or higher difference in volume considered significant.

For the second set of gels, stringent criteria were applied to select spots for quantification and expression profiling. Only spots that were present in both biological duplicate gels of this second set were analyzed by Image Master software. The intensity of matching spots on the gels was compared between the Pro-Q Diamond and SYPRO-Ruby stained gels to detect potential phosphoprotein candidates. If the intensity of the Sypro Ruby stained spot was greater than 50% of the corresponding ProQ Diamond stained spot intensity, the spot was selected as a phosphoprotein. ProQ Diamond nonspecific staining of the nonphosphorylated proteins in the Peppermint Stick MW Standard was nearly undetectable. For spots corresponding to potential phosphoproteins, we compared Pro-Q Diamond intensities between oocytes and eggs in order to determine changes in phosphorylation levels. A similar comparison was made in the SYPRO-Ruby gels to determine quantitative changes in protein abundance between oocytes and eggs. The average normalized volumes for each spot (% of total spot volume) from each sample group were compared, and spots with at least a 1.5-fold differential expression between two samples were considered to be up or down-regulated. With a single exception, spots with changes only in Pro-Q Diamond staining, and not Sypro Ruby staining, were chosen for subsequent protein identification analysis. (The one exception was spot number 1068, which showed a decrease in Sypro Ruby staining from oocyte to egg in addition to staining with Pro-Q Diamond only on the oocyte gels.)

Computer analysis of the gels was performed by the Cornell Proteomics Facility in the Life Sciences Core Laboratories Center.

Relative abundance of proteins within a single spot was estimated by the exponentially modified protein abundance index (emPAI) as described by Yang et al. (2007).

Immobilized Metal Affinity Chromatography (IMAC): 500-600 mature oocytes or unfertilized, activated laid eggs were combined and homogenized in PPIHB (containing 0.1% SDS) as described above. After homogenization, additional PPIHB was added to a final volume of 250 µl. Samples were centrifuged at 20,000 x g for 30 minutes at 4^{o}C and the supernatant was collected. 10 mM DTT (60^{o}C for 30-60 min) was used as a reducing agent and 60 mM iodoacetamide was used for alkylation of cysteine residues. Trypsin was added at a ratio of 1:15 trypsin:total protein and samples were incubated overnight at 37^{o}C. Reactions were stopped with 5% formic acid and samples were acidified to pH 2.5-3.0 with 1 M HCl. Samples were added to equilibrated PHOS-Select Iron Affinity Gel (Sigma) and incubated for a minimum of one hour before the gel was washed to remove unphosphorylated peptide fractions. Phosphopeptides were eluted by adding 200-400 µL Elution Solution (400 mM ammonium hydroxide) for subsequent mass spectrometry analysis.

IMAC enrichment was performed by the Cornell Proteomics Facility in the Life Sciences Core Laboratories Center.

Protein identification: Spots of interest from the 2D gels were picked robotically from a master gel containing both mature oocyte and activated egg samples (Investigator ProPic; Genomic Solutions, Ann Arbor, MI) for in-gel digestion with trypsin and

extraction by robotic ProPrep (Genomic Solutions) using standard protocols (Shevchenko et al., 1996). Spots of interest and IMAC enriched peptides were subjected to nanoLC-ESI-MS/MS analysis on a hybrid triple quadrupole linear ion trap mass spectrometer, the 4000 Q Trap (ABI/MDS Sciex, Framingham, MA). A second mass spectrometry analysis of the IMAC peptides was also performed on a LTQ Orbitrap Velos mass spectrometer (Thermo Scientific, Waltham, MA). The MS/MS data generated from the nanoLC-MS/MS IDA analysis on 2D gel spots and IMAC samples were submitted to Mascot 2.2 for database searching using an in-house licensed Mascot local server. The search was performed to query the NCBInr database (Taxonomy: Drosophila) downloaded from NCBI (July 2008), allowing one missed cleavage site by trypsin. Carbamidomethyl modification of cysteine, methionine oxidation and phosphorylation of serine/threonine/tyrosine were set as variable modifications. All matches that occurred above a 95% confidence interval (CI), with significant scores for the peptides defined by Mascot probability analysis greater than "identity" were considered for the protein identifications. Resulting spectra were inspected manually to verify phosphorylation site identifications. Only peptides with an identified phosphorylation site were included in further analyses. Peptide sequences from the Orbitrap results were manually run through a BLAST analysis (http://blast.ncbi.nlm.nih.gov) to determine the proteins from which they were derived.

Mass spectrometry was performed by the Cornell Proteomics Facility in the Life Sciences Core Laboratories Center.

Characterization of candidate proteins: Function was chosen from the GO terms listed by AmiGO (amigo.geneontology.org). Tissue expression was mined from FlyAtlas (www.flyatlas.org). Unless otherwise indicated, independent evidence for phosphorylation was found at the Uniprot database (www.uniprot.org). In cases where the only evidence for phosphorylation was from Zhai et al. (2008), the protein was considered a known Drosophila phosphoprotein only if the same phosphopeptide was found in our IMAC experiment. If there was no evidence of phosphorylation in Drosophila, homologs were searched in the Uniprot database for reports of phosphorylation, beginning with human and followed by mouse, rat, and Xenopus. Both Uniprot and Pubmed were searched for previously known evidence of phosphorylation. InParanoid (http://inparanoid.sbc.su.se/cgi-bin/index.cgi) gene search was used to identify orthologs in any vertebrate species.

Phosphatase treatment: For Spindly, samples of 75 mature oocytes or 75 eggs were homogenized in 50 µl of modified protease inhibiting buffer containing 1X CIAP Dephosphorylation Buffer (Roche) instead of HEPES, and no Triton X-100. Samples were then split in half. The phosphatase inhibitor Okadaic Acid was added to one half at a concentration of 0.4 µM and calf intestine alkaline phosphatase (*CIAP*, Roche) was added to the other half. Samples were then incubated at 37^{o}C for 15 min. Following the incubation an equal volume of DTT loading buffer was added and samples were boiled and run as described earlier.

For Vap-33-1, lark, and DLIC, 300 mature oocytes or egg were homogenized in 200 µl Extraction Buffer and boiled for 5 minutes. Protein was precipitated by methanol/chloroform and protein pellets were dried overnight. Proteins were

resuspended in 1X Dephosphorylation Buffer (Roche) with 1% SDS and 5% Triton X-100. Samples were split in half and 1/10 the sample volume of CIAP was added to one half. Both CIAP and control samples were incubated at 37 ^{o}C for 1 hour. Protein was once again precipitated with methanol/chloroform. The protein pellet was then resuspended in 1X SDS sample buffer and run as described earlier.

RT-PCR: RNA was extracted from approximately 150 stage 14 mature oocytes collected from *mri* RNAi and control females. 1 μg of RNA was treated with 1 μl RQ1 DNase (Promega) for 37^{o}C for 30 min. The reaction was stopped with 1 μl RQ1 DNase Stop Solution and a 10 min incubation at 65^{o}C. cDNA was synthesized using SMARTScribe Reverse Transcriptase (Clontech) and according to manufacturer's protocol. cDNA from *mri* RNAi and control oocytes was used as the template in a PCR with primers to *mri* or the control gene *RpL32* (which is expect to be present in equal abundance in both samples). Primer sequences for *mri* were 5' – CGA CGG CAT TTC GCA CTT AGT GTT – 3' and 5' – AGG ACA TGT GGA TGA AAG GTC GCT – 3'. *RpL32* primer sequences were 5' – CCG CTT CAA GGG ACA CTA TC – 3' and 5' – GAC AAT CTC CTT GCG CTT CT – 3'. The *mri* primers span an intron, allowing genomic DNA and cDNA to be differentiated by size, to ensure that the observed PCR products did not result from any genomic DNA contamination within the cDNA prep. The reaction was run for 30 cycles. Products were run along with a 1 Kb Plus DNA ladder (Invitrogen) on a 1% agarose gel containing 0.5 μg/ml ethidium bromide.

Fertility of RNAi females: Fly lines with an RNAi construct to the genes of interest were obtained from the Transgenic RNAi Resource Project at Harvard Medical School

(see Table S5 for line numbers). Females from the lines were crossed to nanos-GAL4 males (Bloomington stock 4937) to specifically knockdown the genes in the female germline (referred to as RNAi females). Control females were nanos-GAL4/TM3 siblings from lines that were balanced. Virgin RNAi and control females, aged 3-5 days, were individually mated with wild-type (ORP2) males. Following copulation, males were removed and females were allowed to lay eggs for 24 hours. Every 24 hours, females were transferred to a fresh vial and the number of eggs laid in the previous vial was counted. In this way, the total number of eggs laid by each female over 3 days was determined. After 15 days, the total number of adult progeny in each vial was also counted to determine the fertility of each female. Viability was determined by dividing the total number of adult progeny by the total number of eggs laid and is presented as the average (± standard deviation) for 5 females of each genotype. Exceptions are females knocked down for *eif-4A* for which n = 4, and the control for which n = 13 (summed over 2 experiments).

2.3 Results

2D gel electrophoresis detects dynamic phosphorylation changes:

To visualize proteomic changes that occur upon egg activation in *Drosophila*, we performed two-dimensional (2D) gel analysis of samples containing protein from mature, stage 14 oocytes or 0-30 minute activated, unfertilized laid eggs. We detected 830 protein spots in samples from mature oocytes and 822 protein spots in samples from activated eggs. A subset of the spots detected in mature oocytes were not detected in activated eggs (231/830), suggesting that these proteins are degraded or

undergo post-translational modifications upon egg activation. We also detected protein spots unique to activated eggs (223/822), indicative of translation or post-translational modifications upon activation.

In samples from both stages, approximately 30 percent of the total detected proteins were phosphorylated. This result is similar to the percentage of phosphorylated proteins found in sea urchin unfertilized eggs, and to the percentage of the proteome that has been observed to be phosphorylated in mammalian cells (Ahn and Resing, 2001; Roux et al., 2006; Roux et al., 2008). Of the phosphoproteins we analyzed, 40% were more highly phosphorylated ($\geq$ 2 fold) in mature oocytes and 38% were more highly phosphorylated in the activated egg, indicating that there is a high level of phospho-modulation during egg activation in *Drosophila* (Figure 2.1 and Table 2.1).

IMAC enrichment of phosphopeptides identifies numerous proteins that change in phosphorylation state during egg activation:

After observing the extent of the phosphorylation changes that take place during egg activation by 2D gel, we set out to determine the identities of the proteins that undergo these changes. We applied a peptide-based approach with enrichment of phosphopeptides by immobilized metal affinity chromatography (IMAC). IMAC allowed us to identify a large number of phosphorylated proteins in both mature oocytes and activated eggs; including proteins that may not have been detectable, or appeared differently, on the 2D gels. IMAC also provides the additional advantage that each protein is associated with an identified phosphorylation site.

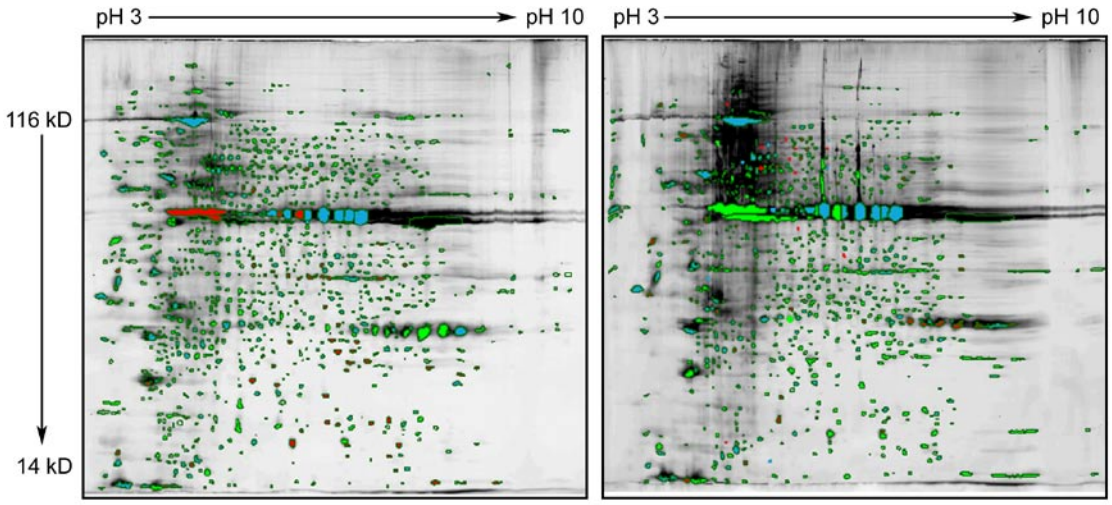

Figure 2.1 ProQ Diamond Phosphorylation stained images of mature oocytes (left) and 0-30 minute activated eggs (right) show global phosphorylation changes take place upon egg activation. The gels were stained with ProQ Diamond Phosphorylation Stain to detect phosphoproteins. Spots are colored to indicate a difference in spot volume between the two samples; a spot with a 2-fold or higher volume in one sample is green, a 2-fold or lower volume in one sample is red, and spots that are approximately the same volume in both samples are blue.

	Unique spots/ Total spots	Phosphorylated at ≥ 0.9	2-fold or higher Phosphorylation
Mature oocytes	231/830 (27.8%)	163/555 (29.4%)	46/114 (40.4%)
Activated eggs	223/822 (27.1%)	112/405 (27.6%)	43/112 (38.4%)

Table 2.1 Changes to the proteome and phosphoproteome upon egg activation in *Drosophila* observed by 2D gel analysis. Colloidal Coomassie Blue (CCB) and ProQ stained images of mature oocytes and activated eggs were analyzed for total number of proteins, phosphoproteins, and phosphoprotein changes. The left column shows the number of unique spots out of the total number of spots stained with CCB in the indicated samples. The middle column shows the number of spots in the indicated sample that were considered phosphorylated out of the total number of spots that could be analyzed (see Materials and Methods). The far right column shows that number of spots that were more highly phosphorylated in the indicated sample, out of the total number of spots that could be analyzed. Analysis and table are from the dissertation of V.L. Horner.

Comparisons were made between the phosphopeptides that we found in either mature oocyte or activated egg samples, resulting in a total of 273 phosphopeptides that were identified in only one sample or the other; 205 in the mature oocyte and 68 in the activated egg. In most cases the phosphorylation(s) of a given protein was found within a single peptide sequence, although we also identified a few proteins phosphorylated within multiple phosphopeptide sequences. Thus, these 273 unique phosphopeptides define 238 proteins: 174 with mature oocyte specific phosphorylations, 58 with activated egg specific phosphorylations, and 6 proteins that were phosphorylated in both samples, but at different sites. We consider these candidates for proteins that change in phosphorylation state during egg activation. Our finding of more phosphorylated peptides in mature oocytes than in activated eggs also suggests that egg activation is accompanied by more dephosphorylation events than phosphorylation events.

Since the IMAC experiment does not control for changes in protein abundance, some of the changes observed could be due to protein translation or degradation instead of a change in phosphorylation state. However, we believe that majority of the changes we identified represent true changes in phosphorylation state. Among the phosphoproteins identified only in the mature oocyte samples are GNU and ERK (encoded by the *rolled* gene), two proteins previously known to be dephosphorylated during egg activation (Renault et al., 2003; Sackton et al., 2007), demonstrating our ability to detect relevant phosphorylation changes by this method. In addition, data from previous mass spectrometry experiments informed us that for approximately

25% of the proteins identified by IMAC the protein is present in both oocytes and activated eggs (unpublished results).

Additional proteins that change in phosphorylation state are identified from 2D gels:

Recognizing that any single proteomic method is not saturating for the proteome, we ran a second set of 2D gels in an effort to identify additional proteins undergoing phosphorylation changes. While we were unable to control for abundance changes in the IMAC experiment, we chose to control for it here. We applied strict criteria for selecting spots for identification and selected spots that only showed differences between oocytes and activated eggs in phosphorylation (ProQ Diamond) but not protein abundance (SYPRO-Ruby). Even though these criteria reduced the number of proteins that we identified from the 2D gels, they ensured that the spot changes we focused on were specifically due to phosphorylation differences between the samples. Thirty spots met these criteria; eighteen of these represented proteins that were more highly phosphorylated in the mature oocyte, while 12 were more highly phosphorylated in activated eggs (Figure 2). Mass spectrometry of the selected spots resulted in a total of 83 protein IDs, as most spots contained more than one protein (Table 2.2). We hypothesize that the phosphorylation change of a given spot is most likely due to the most highly abundant protein present within the spot and/or the proteins for which we identified a phosphorylated peptide. However, it is possible that multiple co-migrating proteins contributed to the observed ProQ Diamond staining. We therefore considered all proteins identified as candidate proteins that may be changing in phosphorylation state during egg activation.

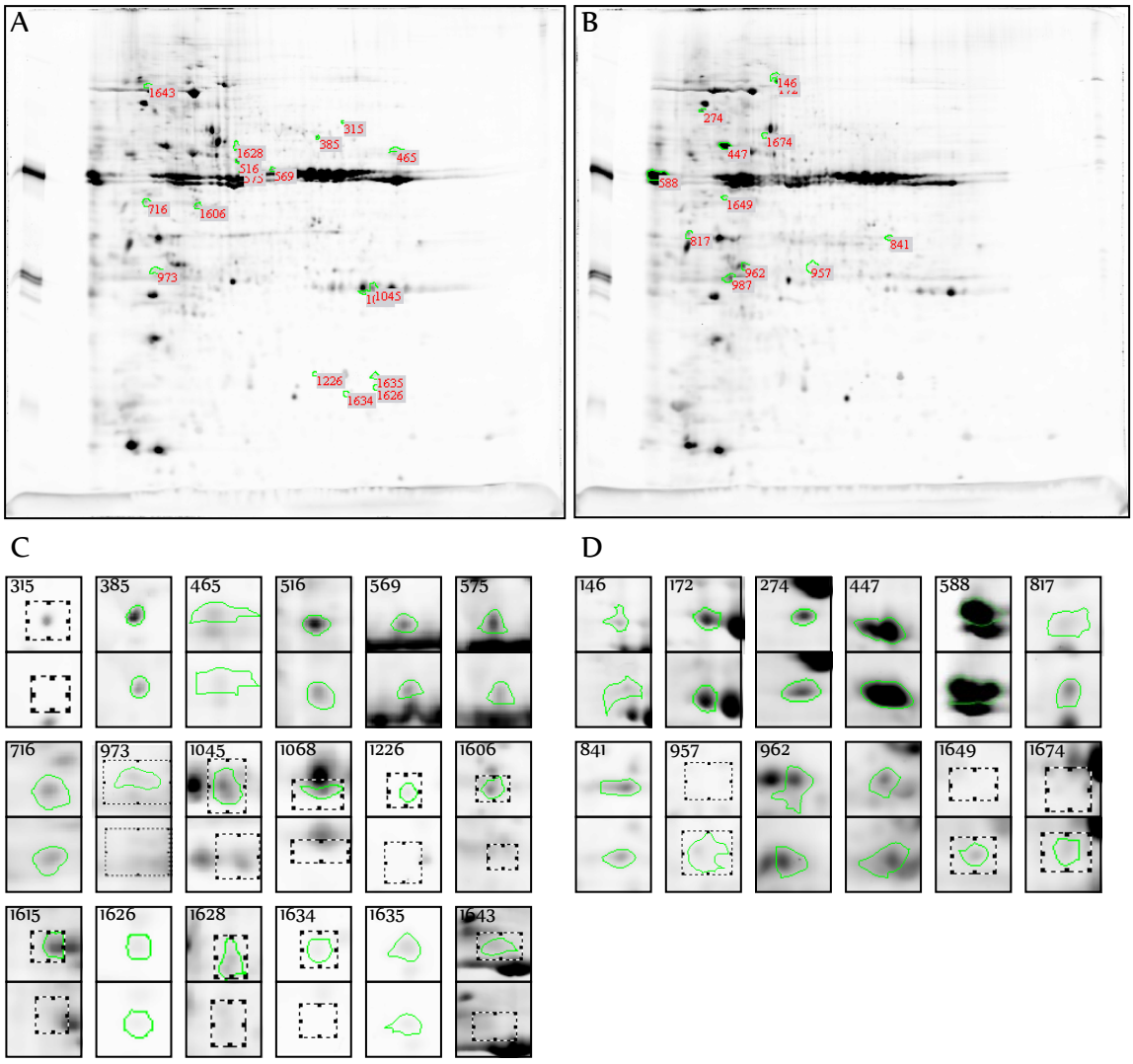

Figure 2.2 2D gels stained with ProQ Diamond phosphorylation stain and spots chosen for identification. Gels are shown for A) mature oocyte proteome and B) unfertilized activated egg proteome. Spots chosen for identification by mass spectrometry are indicated with their corresponding spot ID#. Images of each individual spot chosen for identification are shown below for C) spots that were upregulated or unmatched in the mature oocyte and D) spots that were upregulated or unmatched in the activated egg.

Table 2.2 Proteins identified from 2D gels as changing in phosphorylation state during Drosophila egg activation. Proteins for which a phosphopeptide was found are marked with a Y in the phosphopeptide column and the relative abundance of a protein within a single spot is given. Spots in red (starting at Spot ID 146) were more highly phosphorylated in the activated egg.

Spot ID	CG	Protein(s)	Phospho-peptide	Relative Abundance (%)
315	CG17246	Succinate dehydrogenase A	N	100
385	CG8439	T-complex Chaperonin 5	Y	98
	CG8351	Tcp-1eta	N	1
	CG5384		N	1
465	CG14309		N	50
	CG3590		N	28
	CG13425	bancal	N	15
	CG31293	recombination-defective	Y	7
516	CG8209		Y	49
	CG11154	ATP synthase beta subunit	N	25
	CG6546	Brahma associated protein 55kD	N	9
	CG9556	alien	N	7
	CG7997		N	5
	CG4535	FK506-binding protein FKBP59	N	3
		actin	N	2
569	CG9075	eukaryotic translation initiation factor 4A	N	73
	CG2985	yolk protein 1	N	14
	CG13349		N	6
	CG2915		N	4
	CG5330	Nucleosome assembly protein 1	N	3
575	CG9075	eukaryotic translation initiation factor 4A	N	68
	CG2979	yolk protein 2	N	20
	CG16916	Rpt3	N	6
	CG2985	yolk protein 1	N	6
716	CG4930	Endonuclease G inhibitor	N	86
	CG4299	Set	N	14
973	CG3183	geminin	N	57
	CG8582	Sh3beta	N	43
1045	CG4183	Heat shock protein 26	N	50
	CG13922	mitochondrial ribosomal protein L46	N	37
	CG1721	Phosphoglyceromutase	N	13
1068	CG1274	thioredoxin peroxidase 2	N	84
	CG4466	Heat shock protein 27	N	9
	CG7490	Ribosomal protein LP0	N	7

1226	CG3612	bellwether	N	100
1606	CG9946	eukaryotic translation initiation factor 2alpha	N	91
	CG10230	Rpn9	N	7
	CG1906	alphabet	N	2
1615	CG11154	ATP synthase beta subunit	N	56
1615	CG11844	vig2	Y	18
	CG3612	bellwether	N	15
	CG8209		Y	11
1626	no ID			
1628	CG7070	pyruvate kinase	N	30
	CG8308	alpha-Tubulin at 67C	N	18
	CG1799	raspberry	N	11
	CG2263		N	11
	CG7085	lethal (2) s5379	N	9
	CG5330	Nucleosome assembly protein 1	N	7
	CG1938	Dynein light intermediate chain	N	5
	CG5525		N	5
	CG5320	Glutamate dehydrogenase	N	4
1634	CG10682	vihar	N	64
	CG9705		N	36
1635	CG9705		N	100
1643	CG13388	A kinase anchor protein 200	N	96
	CG1242	Heat shock protein 83	N	4
146	CG6603	Hsc70Cb	Y	72
	CG5520	Glycoprotein 93	N	15
	CG4206	Minichromosome maintenance 3	N	13
172	CG2331	TER94	Y	91.5
	CG7528	Smt3 activating enzyme 2	Y	5.5
	CG6603	Hsc70Cb	N	2
	CG1242	Heat shock protein 83	N	1
274	CG4264	Heat shock protein cognate 4	N	48
	CG8223		Y	38
	CG9050	palisade	N	8
	CG6751		N	6
447	CG5330	Nucleosome assembly protein 1	N	98
	CG8439	T-complex Chaperonin 5	N	1.5
	CG8351	Tcp-1eta	N	0.5
588	CG2985	yolk protein 1	N	39
	CG2979	yolk protein 2	N	38
	CG11129	yolk protein 3	N	17
		actin	N	4
	CG3127	Phosphoglycerate kinase	N	1
	CG5880		N	1
817	CG2207	Decondensation factor 31	Y	98
	CG8893	Glyceraldehyde 3 phosphate dehydrogenase 2	N	1.5

	CG18174	Rpn11	N	0.5
841	CG7490	Ribosomal protein LP0	N	100
957	CG18190		N	100
962	CG4912	eEF1delta	Y	51
	CG12131	Adam	N	31
	CG31196	14-3-3epsilon	N	16
962	CG7490	Ribosomal protein LP0	N	1
	CG16725	survival motor neuron	N	1
987	CG4912	eEF1delta	N	54
	CG17870	14-3-3zeta	N	29.5
	CG9273	Replication protein A2	N	7
	CG8947	26-29kD-proteinase	N	6
	CG9154		N	2
	CG5921		N	0.5
1649	CG7967		N	32
	CG6692	Cysteine proteinase-1	N	29
	CG5094	small glutamine-rich tetratricopeptide containing protein	N	15
	CG13630		N	13
	CG8309	Transport and Golgi organization 7	N	11
1674	CG17291	Protein phosphatase 2A at 29B	N	74
	CG1242	Heat shock protein 83	N	7
	CG32190	NUCB1	N	6.5
	CG3223		N	6.5
	CG2774		N	6

Only ten proteins were identified by both the 2D gels and IMAC. Thus, neither method was saturating for the proteome. For six of these proteins (Vig2, DLIC, Hsp26, Smt3 activating enzyme 2, CG8209, and CG14309) the results were consistent between the two methods. The other four proteins (Nucleosome assembly protein 1, Yolk protein 1, A kinase anchor protein 200, and CG18190) were either identified in multiple spots on the 2D gels or were identified from spots more highly phosphorylated in the opposite sample from which we identified a phosphopeptide by IMAC. It is possible that these proteins have additional phosphorylation sites that we failed to identify by IMAC and the overall change in phosphorylation state is in the direction indicated by the 2D gels. Additional studies will be required to fully define the phosphorylation state of these proteins in oocytes and activated eggs.

Western blots confirm phosphorylation state change for select proteins:

The phosphorylation of a protein can affect its electrophoretic mobility through a gel: A more highly phosphorylated protein will often have a slower mobility than a less phosphorylated form of the same protein. This difference in mobility can be enhanced by the addition of Phos-tag, which binds the phosphate groups on phosphorylated proteins, slowing their movement through a polyacrylamide gel (Kinoshita et al., 2006). Thus, if a protein is present in different phosphorylation states between samples, this may appear as a band shift on a Western blot.

We tested a subset of seven of our IMAC candidates with available antibodies by Western blot to see if we could observe a shift for any of these proteins when comparing samples from mature oocytes and activated eggs. We observed a shift for five candidates - lark, Dynein light intermediate chain (DLIC), Spindly, Spd-2, and

Vap-33-1 (Figure 2.3). Treatment of the samples with a phosphatase (CIAP) confirms that the shifts seen on the Western blot are due to phosphorylation differences (Figure 2.4). Because phosphorylation does not guarantee a change in electrophoretic mobility, our failure to observe a shift for the other two candidates (Cup and Slender lobes) does not rule out that they are phosphoregulated.

Spindly, DLIC, Spd-2, and lark show shifts indicative of dephosphorylation during egg activation and Vap-33-1 appears to exist in an intermediate phosphorylation state that is oocyte specific. Spindly protein migrates more slowly in mature oocytes than in activated eggs, indicating that a more highly phosphorylated form of the protein is present in the oocyte (Figure 2.3A). DLIC protein runs as a doublet, the upper band of which was shown by Mische et al. (2008) to represent a phosphorylated form of the protein. We observe this doublet in mature oocytes, however only the lower band is detectable in unfertilized activated eggs (Figure 2.3B). Spd-2 protein appears as a smear in the oocyte lane when run on a Phos-tag gel (5% acrylamide, 3.5 μM Phos-tag), indicating that the protein is phosphorylated. This smear is no longer present in the activated egg lane, consistent with dephosphorylation of Spd-2 during egg activation (Figure 2.3C). Phos-tag gels (10.6% acrylamide, 25 μM Phos-tag) also allow us to visualize slower mobility fractions of lark and Vap-33-1 proteins which correspond to phosphorylated forms of the proteins. For lark, the phosphorylated state is specific to the mature oocyte, again consistent with dephosphorylation of the protein during egg activation (Figure 2.3D). Vap-33-1 protein appears to exist in two distinct phosphorylation states, one of which is oocyte specific (Figure 2.3E). These results are consistent with our identification of mature

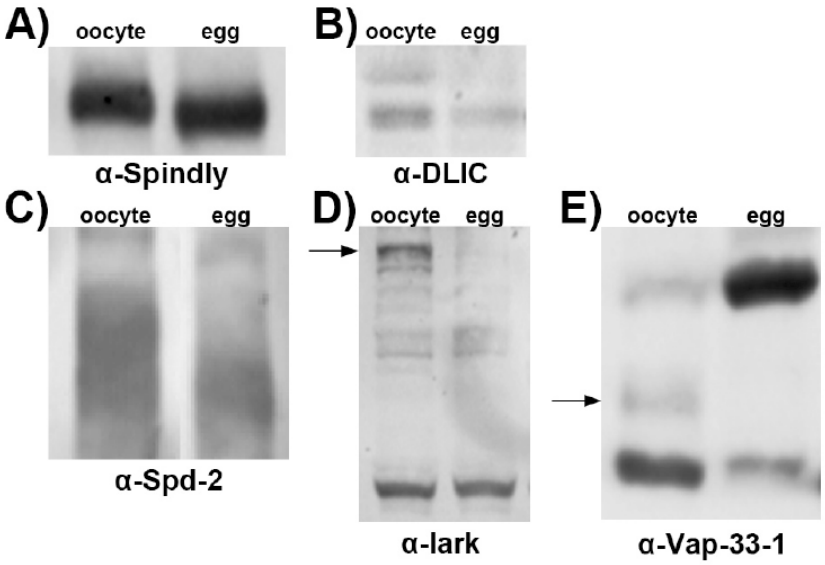

Figure 2.3 Western blots confirm a change in phosphorylation state for A) Spindly B) DLIC C) Spd-2 D) lark and E) Vap-33-1 upon egg activation. Oocyte specific phosphorylation states of lark and Vap-33-1 are indicated by arrow.

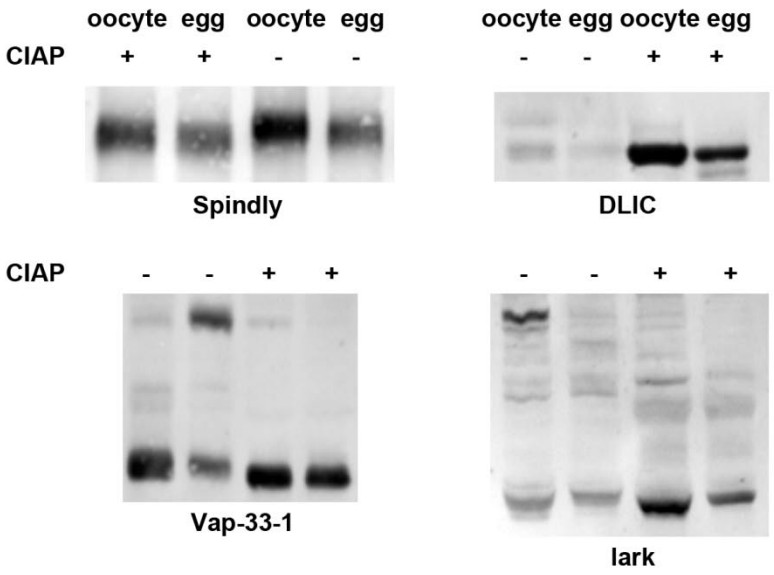

Figure 2.4 Phosphatase treatment confirms that band shifts are due to phosphorylation.

oocyte specific phosphopeptides for these five proteins, supporting the validity of the candidates we have identified.

RNAi knockdown of candidates identifies a new gene important for the first embryonic mitosis:

We next used RNAi to test our hypothesis that our candidates include new genes that act during egg activation or embryogenesis. We attempted to knock down 18 of our candidates by female germline-specific RNAi and found that 6 are essential for female fertility and that knockdown of a seventh severely reduced fertility (only 33% of laid eggs produced adult progeny)(Table 2.3). The lack of a phenotype for the remaining 11 candidates indicates that maternal expression of these genes is either not essential for early development, perhaps due to redundant molecules, or that we failed to successfully knock down the genes by RNAi. Of the 6 genes whose knockdown caused sterility, knockdown of 4 prevented the production of eggs, indicating roles for these genes in oogenesis. Knockdown of the other 2 resulted in eggs that were defective in embryogenesis. Knockdown of one of these genes, *PyK*, leads to embryos that are able to complete early embryonic mitosis (data not shown), possibly due to insufficient knockdown to reveal earlier functions of the protein. In contrast, we find the early arrest caused by knockdown of the other gene, *mrityu* (*mri*), to be of particular interest.

Mri, which our IMAC experiments detected as a protein that is phosphorylated during egg activation, is a BTB/POZ domain-containing protein with no previously known function (flybase.org). *mri* RNAi females lay eggs, but those eggs fail to hatch (Supplementary Table 2.4). RT-PCR confirms that *mri* is knocked down in mature

Table 2.3 Viability of laid eggs when candidate genes are knocked down by female germline specific RNAi. Genes were knockdown using a nanos-GAL4 driver. Viability is presented as the percentage of eggs laid that resulted in adult progeny (± standard deviation). N=5, except for *eif-4A* and control (see Supplementary Methods for details).

Knockdown gene	**TRiP line #**	**Viability (%)**
CG7597	HMS00155	no oocytes
CG9609	HMS01000	no oocytes
CG5602	HMS01036	no oocytes
Rtf1	HMS00168	no oocytes
PyK	GL00099	0 (± 0)
mrityu	GL00033	0 (± 0)
Pk92B	GL00238	33 (± 18)
D1	HMS00061	84 (± 9.0)
Eps-15	HMS00947	98 (± 1.3)
HIP-R	HMS00988	96 (± 1.6)
5-HT1A	HMS00823	98 (± 1.9)
rasputin	HMS00269	98 (± 2.1)
Sodh-1	HMS01028	98 (± 0.73)
RPA2	HMS01061	86 (± 26)
Apc	HMS 00188	92 (± 11)
RhoGAP92B	HMS00268	99 (± 1.5)
myopic	HMS00836	94 (± 4.1)
eif-4A	HMS00927	99 (± 0.60)
Control (no knockdown)		97 (± 2.5)

Table 2.4 Females with *mriytu* knocked-down in the germline produce eggs, but no progeny. Egg and progeny counts are the totals over three days, presented as the average of 5 females (± standard deviation).

	# eggs	# progeny
Control n = 5	107 (± 14.9)	105 (± 15.2)
mrityu RNAi n = 5	141 (± 23)	0

oocytes of RNAi females, indicating that *mri* expression in the germline is necessary for female fertility (Figure 2.5). To determine if the embryos laid by *mri* RNAi mothers arrested early or late in embryogenesis we stained 1-3 hr old embryos with DAPI to observe the number of nuclei present. We failed to observe more than 5 nuclei in any *mri* arrested embryos (n = 30), indicating an early arrest point. To confirm that embryos from *mri* RNAi females are fertilized prior to their developmental arrest, we stained 0-90 minute old embryos with an anti-sperm tail antibody. A sperm tail was clearly visible within both control and RNAi embryos (7/8 control; 11/12 RNAi), indicating that the sterility of *mri* RNAi females is not due to a lack of fertilization (Figure 2.6A).

To determine the specific arrest point of the RNAi embryos, we then stained them with propidium iodide and anti-tubulin antibody, to visualize DNA and microtubules, respectively. While 1-3 hour old control embryos have undergone multiple rounds of syncytial mitosis (Figure 2.6B), we never observe such divisions in *mri* RNAi embryos. Instead, these embryos arrest during metaphase of the first 1-3 mitotic divisions (Figure 2.6C and Figure 2.7). In approximately half of the embryos observed (14/30) only one nucleus is present within the egg, while another 30% (9/30) contain only 2 nuclei. Our ability to find a polar body in one-third of these embryos suggest that meiosis able to resume and complete (Figure 2.6D), leading to our hypothesis that the arrest is during the first or second embryonic mitosis. However, we can not currently rule out the possibility that we are observing either the single male pronucleus, or the male and female pronuclei. Additionally, we observed two embryos that appear to still be arrested in meiosis I. We attribute the slight variability

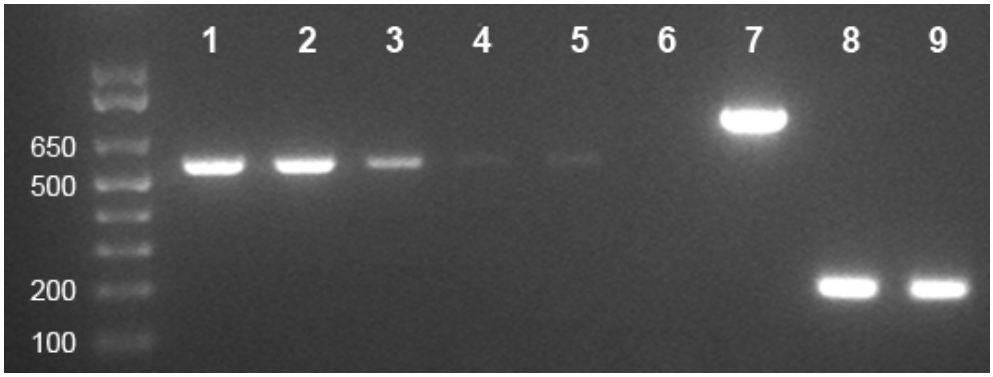

Figure 2.5 RT-PCR confirms that *mrityu* is knocked-down in mature oocytes. PCR was run with primers for *mri* unless otherwise indicated. Lanes:1) 100% control cDNA 2) 50% control cDNA 3) 10% control cDNA 4) 1% control cDNA 5) 100% *mrityu* RNAi cDNA 6) no template 7) genomic DNA 8) 100% control cDNA with *RpL32* primers 9) 100% *mrityu* RNAi cDNA with *RpL32* primers.

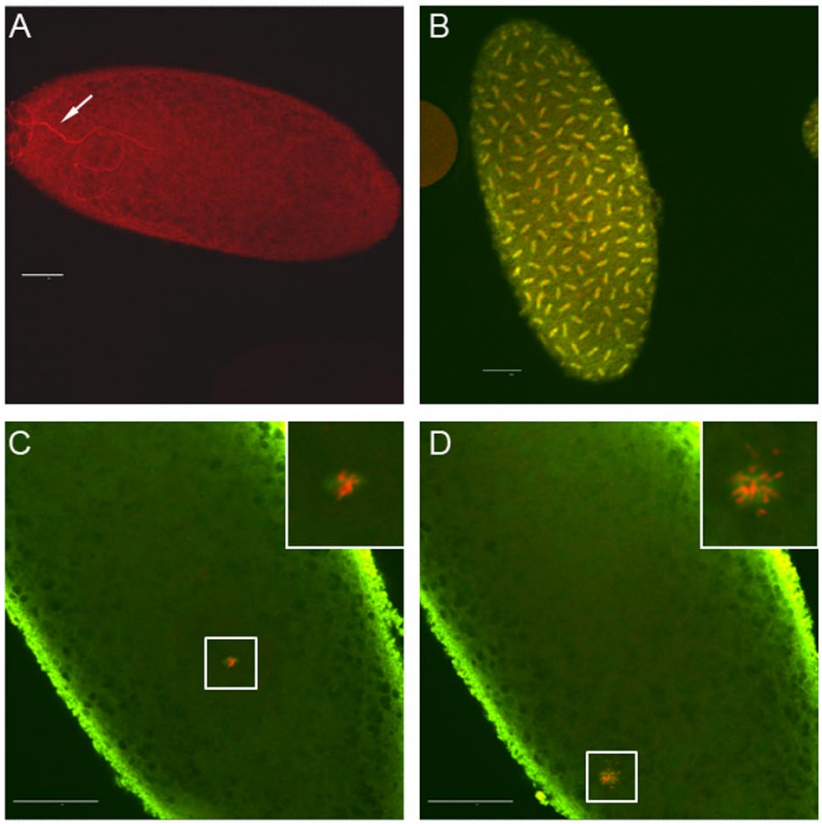

Figure 2.6 Embryos are fertilized but arrest early when *mrityu* is knocked-down in the mother. A) Anti-sperm tail antibody (red) was used to visualize the sperm within 0-90 minute embryos laid by *mri* RNAi females. B-D) One to three hour old embryos laid by *mri* RNAi or control females were stained with propidium iodide to visualize DNA (red) and anti-tubulin antibody to visualize microtubules (green). Control embryos undergo mitotic divisions (B) while RNAi embryos typically arrest with a single nucleus (C). The visualization of a polar body rosette (D) suggests that meiosis is able to complete in these embryos. Insets are zoomed in images of the boxed region.

Scale bar = 50 μm

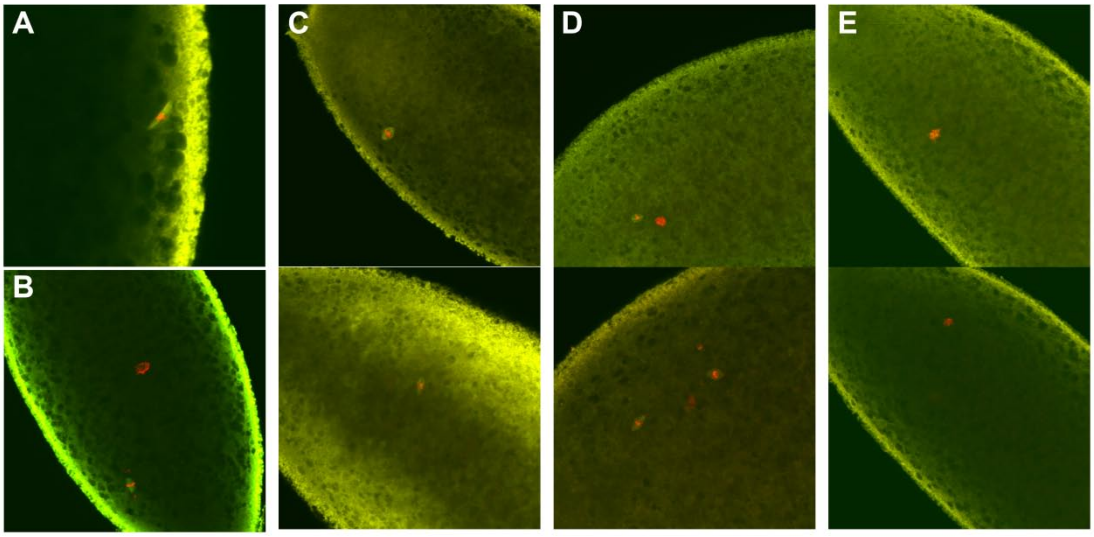

Figure 2.7 Additional arrest points observed for *mrityu* RNAi embryos. A) meiosis I B) single nucleus and possible second nucleus with no associated tubulin C) two nuclei D) 4-5 nuclei E) polar body and one possible nucleus with no associated tubulin. C-E each show one embryo at two different focal planes.

in the arrest point to the variable efficiency of RNAi and the fact that we are able to reduce, but not eliminate, the *mri* transcript in the oocyte. Further studies of *mrityu* will benefit from the creation of a mutant line that eliminates all Mrityu protein. This will allow us to pinpoint the arrest point and more fully characterize the mechanism by which *mrityu* contributes to the regulation of early embryonic development.

By screening a small subset of the phosphoproteins we identified in this study, we have found a new gene, *mrityu,* that is required for early embryonic development. These findings indicate that the phosphoregulated proteins we identified can be a rich source of molecules needed for the oocyte-to-embryo transition and that further RNAi screening will reveal additional genes that act during this early developmental period.

2.4 Discussion

Egg activation is a rapid and highly regulated set of events that allow a relatively quiescent oocyte to transition to the cellular state necessary for embryo development. Much work has been done to understand the initial calcium signal that triggers egg activation in most organisms, and a few of the immediate downstream molecules that transduce this calcium signal have been identified, primarily the kinase CaMKII and the phosphatase calcineurin (Backs et al., 2010; Chang et al., 2009; Takeo et al., 2010). In addition, we know the ultimate cellular events that occur when egg activation is successful – modifications of the egg coverings, resumption and completion of meiosis, translation of new proteins, and degradation of specific maternal macromolecules [reviewed in (Horner and Wolfner, 2008)]. However, the

pathways and proteins that connect the early triggers of activation to the final downstream events remain largely unknown.

We have identified a set of proteins that will help us to better understand the connections between the early triggers and final events of egg activation. As this transition occurs before zygotic transcription and too early for major effects of any new protein synthesis, we explored the possibility that post-translational modifications might activate or inactivate critical molecules during egg activation. Identifying the molecules that are regulated during this transition will provide new molecular insights into this important developmental period, as well as new candidates to test for roles in egg activation. Here we report that large-scale phosphoproteome changes occur during *Drosophila* egg activation. We identified a total of 311 proteins that change in phosphorylation state during this transition; these include proteins that we found to be phosphorylated in only mature oocytes or activated eggs, as well as proteins that changed in the specific phosphorylation site between samples.

Our results show the importance of combining proteomic approaches and the complementary, rather than redundant, nature of these methods; consistent with studies that compared protein-based and peptide-based proteomic methods (Wu et al., 2006). The 2D gels provide a global view of the protein differences found between the two states and do not require the specific phosphorylated peptide of a protein to be detectable by mass spectrometry. In contrast, IMAC does require the identification of the phosphorylated peptide, but does not depend on the protein being detectable, or appearing differently, on a gel. Thus, by combining methods with different strengths we were able to expand the number, and type, of proteins within our data set. Even so,

we do not expect that the 311 proteins we identified represent the full list of proteins that are phospho-regulated during egg activation. However, they provide an important start in providing new candidates to test for roles in egg activation and improving our understanding of the importance of phosphorylation during this period of development. Through a pilot genetic screen, which identified *mrityu* as a new gene with an essential role in early *Drosophila* development, we verified that the phospho-modified proteins we detected will be a source of new regulators of this process.

Large scale phosphorylation changes occur during egg activation

We showed here that a relatively large fraction of the *Drosophila* oocyte proteome (~30%) is phospho-modified during egg activation. Of the 311 phospho-modulated proteins that we identified by proteomic comparison of mature oocytes (prior to egg activation) and activated eggs, we found 84 *Drosophila* proteins, and the *Drosophila* homologs of another 123 proteins from other organisms, that were known to be phospho-modified in at least one cell type. The proteins that we identified include two (ERK and GNU) that had previously been shown to be phospho-modified during this time-window (Sackton et al.,2007; Renault et al., 2003) and five more (Spd2, Spindly, lark, DLIC, and Vap33) whose phosphorylation changes during egg activation we verified independently by Western blotting.

The proteins identified in this study fall into a number of functional classes (based on known and/or predicted functions) (Table 2.5). These include calcium binding/regulation, cell cycle, metabolism/glycolysis, proteolysis, and protein translation; all classes which are anticipated to act during egg activation. Our finding

Table 2.5 All proteins identified from 2D gels and IMAC experiments. Each protein is described by (A) function and presence of vertebrate homologs and (B) expression, previously shown evidence of phosphorylation, method (2D-gel or IMAC) by which it was identified in this study, and the sample (mature oocyte or activated egg) that it was found to be phosphorylated in. NA for expression indicates no conclusive data available on Flyatlas. Y^D indicates evidence of phosphorylation in Drosophila.

Table 2.5A CG	Protein Name	Function	Conserved in Vertebrates
CG31196	14-3-3epsilon	Ras protein signal transduction	Y
CG17870	14-3-3zeta	Ras protein signal transduction	Y
CG8947	26-29kD-proteinase	proteolysis	Y
CG13388	A kinase anchor protein 200	protein kinase A binding	N
	actin	structural constituent of cytoskeleton	Y
CG5712	ACXD	adenylate cyclase activity	N
CG12131	Adam	translation initiation factor	Y
CG1070	alhambra	regulation of transcription	Y
CG9556	alien	negative regulation of transcription	Y
CG4260	alpha-Adaptin	protein transporter activity	Y
CG1906	alphabet	phosphatase activity	Y
CG8308	alpha-Tubulin at 67C	pronuclear migration	N
CG2446	Amun	protein binding	Y
CG6631	anastral spindle 1	mitotic spindle organization	Y
CG1451	APC-like	microtubule binding	Y
CG11154	ATP synthase beta subunit	proton transport	Y
CG6137	aubergine	RNA binding	N
CG6877	Aut1	autophagy	Y
CG7926	axin	beta-catenin binding	Y
CG6386	ballchen	protein kinase activity	Y
CG13425	bancal	RNA binding	Y
CG5529	BarH1	regulation of transcription	Y
CG3612	bellwether	proton transport	Y
CG8276	bicoid-interacting protein 3	regulation of transcription	Y
CG6546	Brahma associated protein 55kD	mitosis	Y
CG10542	Bre1	histone modification	Y
CG9097	bric a brac 1	regulation of transcription	Y
CG42344	bruchpilot	calcium channel activity	Y

Table 2.5A (continued)

CG	Protein Name	Function	Conserved in Vertebrates
CG31132	BRWD3	phagocytosis	Y
CG12892	Caf1-105	DNA binding	Y
CG11958	calnexin	protein folding	Y
CG12019	Cdc37	protein tyrosine kinase activator activity	Y
CG7597	Cdk12	cyclin-dependent protein kinase activity	Y
CG7464	chitin synthase 2	chitin synthase activity	Y
CG4145	collagen type IV	extracellular matrix structural constituent	Y
CG33957	cp309	microtubule nucleation	Y
CG34389	crossveinless c	Rho GTPase activator activity	Y
CG11181	cup	regulation of translation	N
CG6081	Cyp28d2	electron carrier activity	N
CG6692	Cysteine proteinase-1	proteolysis	Y
CG9745	D1 chromosomal protein	satellite DNA binding	N
CG2207	Decondensation factor 31	histone binding	N
CG2175	defective chorion 1	structural constituent of chorion	N
CG42799	dikar	unknown	Y
CG1938	Dynein light intermediate chain	mitotic spindle organization	Y
CG8598	eco	mitotic spindle organization	Y
CG4912	eEF1delta	translation elongation factor	Y
CG9841	EfSec	translation elongation factor	Y
CG6542	Egg-derived tyrosine phosphatase	protein phosphatase activity	Y
CG10315	eIF2B-delta	translation initiation factor	Y
CG32859	eIF4E-7	translation initiation factor	Y
CG10840	eIF5B	translation initiation factor	Y
CG5345	Eip55E	catalytic activity	Y
CG4930	Endonuclease G inhibitor	ribonuclease inhibitor activity	N

Table 2.5A (continued)

CG	Protein Name	Function	Conserved in Vertebrates
CG16932	Eps-15	calcium ion binding	Y
CG10837	eukaryotic initiation factor 4B	translation initiation factor	Y
CG9946	eukaryotic translation initiation factor 2alpha	translation initiation factor	Y
CG9075	eukaryotic translation initiation factor 4A	translation initiation factor	Y
CG10192	eukaryotic translation initiation factor 4G2	translation initiation factor	N
CG15573	femcoat	structural constituent of chorion	N
CG4535	FK506-binding protein FKBP59	regulation of calcium ion transport	Y
CG9611	flyers-cup	Ras protein signal transduction	Y
CG7004	four wheel drive	1-phosphatidylinositol 4-kinase activity	Y
CG11086	Gadd45	JNK cascade	Y
CG3183	geminin	negative regulation of DNA replication	Y
CG5272	giant nuclei	regulation of cell cycle	N
CG5320	Glutamate dehydrogenase	glutamate dehydrogenase activity	Y
CG8893	Glyceraldehyde 3 phosphate dehydrogenase 2	glycolysis	Y
CG7254	glycogen phosphorylase	glycogen phosphorylase activity	Y
CG5520	Glycoprotein 93	protein folding	Y
CG8384	groucho	negative regulation of transcription	Y
CG4183	Heat shock protein 26	protein binding	Y
CG4466	Heat shock protein 27	protein refolding	Y
CG1242	Heat shock protein 83	protein folding	Y
CG4264	Heat shock protein cognate 4	protein folding	Y
CG4261	helicase 89B	DNA binding	Y
CG7269	helicase at 25E	mitotic spindle organization	Y
CG3373	hemomucin	RNA binding	Y
CG4451	Heparan sulfate 6-O-sulfotransferase	sulfotransferase activity	Y
CG12749	heterogeneous nuclear ribonucleoprotein at 87F	RNA splicing	Y

Table 2.5A (continued)

CG	Protein Name	Function	Conserved in Vertebrates
CG10473	hook-like	protein targeting to lysosome	Y
CG2947	Hsc/Hsp70-interacting protein related	protein folding	Y
CG6603	Hsc70Cb	protein folding	Y
CG3504	inaD	calmodulin binding	Y
CG10101	Ionotropic receptor 84a	glutamate binding	N
CG3654	Jarid2	DNA binding	Y
CG1804	kek6	unknown	Y
CG6217	knickkopf	chitin biosynthetic process	N
CG32464	l(3)82Fd	unknown	Y
CG42551	la related protein	spindle assembly	Y
CG8597	lark	RNA splicing	Y
CG8465	lethal (1) G0222	unknown	Y
CG7085	lethal (2) s5379	unknown	Y
CG5738	lola like	regulation of transcription	Y
CG18543	matrimony	female meiosis	N
CG7162	mediator complex subunit 1	regulation of transcription	Y
CG3697	meiotic 9	female meiosis	Y
CG42572	microcephalin	mitosis	Y
CG4206	Minichromosome maintenance 3	chromatin binding	Y
CG16973	misshapen	JNK cascade	Y
CG13922	mitochondrial ribosomal protein L46	structural constituent of ribosome	Y
CG18124	mitochondrial transcription termination factor	termination of mitochondrial transcription	N
CG32491	modifier of mdg4	chromatin binding	Y
CG2050	modulo	DNA binding, RNA binding	N
CG18740	moira	regulation of transcription	Y
CG1216	mrityu	protein homooligomerization	Y

Table 2.5A (continued)

CG	Protein Name	Function	Conserved in Vertebrates
CG11156	mutagen-sensitive 101	oogenesis	Y
CG9311	myopic	protein phosphatase activity	Y
CG32156	myosin binding subunit	myosin phosphatase activity	Y
CG3845	NAT1	translation initiation factor	Y
CG17704	Nipped-B	regulation of transcription	Y
CG17255	no circadian temperature entrainment	circadian clock	Y
CG32190	NUCB1	calcium ion binding	Y
CG5330	Nucleosome assembly protein 1	regulation of transcription	Y
CR9700	Odorant receptor 85e	odorant binding	N
CG5581	otefin	negative regulation of transcription	N
CG6824	ovo	regulation of transcription	N
CG9050	palisade	eggshell formation	N
CG33103	papilin	extracellular matrix structural constituent	Y
CG4845	phagocyte signaling impaired	immune response	Y
CG3127	Phosphoglycerate kinase	glycolysis	Y
CG1721	Phosphoglyceromutase	glycolysis	Y
CG9183	plutonium	regulation of cell cycle	Y
CG8169	Pms2	protein binding	Y
CG4532	pod1	actin binding	Y
CG7913	PP2A-B'	protein phosphatase regulator	Y
CG30342	pre-mRNA processing factor 38	RNA splicing	Y
CG4904	proteasome 35kD subunit	proteolysis	Y
CG1519	proteasome alpha7 subunit	proteolysis	Y
CG10149	proteasome p44.5 subunit	proteolysis	Y
CG4720	protein kinase at 92B	protein kinase activity	Y
CG2049	protein kinase related to protein kinase N	protein kinase activity	Y
CG17291	Protein phosphatase 2A at 29B	phosphatase activity	Y

Table 2.5A (continued)

CG	Protein Name	Function	Conserved in Vertebrates
CG9181	protein tyrosine phosphatase 61F	protein phosphatase activity	Y
CG7752	putzig	zinc ion binding	Y
CG7070	pyruvate kinase	glycolysis	Y
CG5627	rab3-GEF	activation of MAPK activity	Y
CG1799	raspberry	IMP dehydrogenase activity	Y
CG9412	rasputin	Ras protein signal transduction	Y
CG31293	recombination-defective	DNA replication	Y
CG9273	Replication protein A2	DNA replication	Y
CG40494	RhoGAP1A	regulation of Rho protein signal transduction	Y
CG6811	RhoGAP68F	signal transduction	Y
CG4755	RhoGAP92B	signal transduction	Y
CG9774	Rho-kinase	protein kinase activity	Y
CG5371	ribonucleoside diphosphate reductase large subunit	DNA replication	Y
CG7434	ribosomal protein L22	translation	N
CG1821	ribosomal protein L31	translation	Y
CG7490	Ribosomal protein LP0	structural constituent of ribosome	Y
CG4420	rings lost	proteolysis	Y
CG12559	rolled	protein kinase activity	Y
CG18174	Rpn11	proteolysis	Y
CG10230	Rpn9	proteolysis	Y
CG16916	Rpt3	proteolysis	Y
CG10955	Rtf1	transcription initiation	Y
CG11427	ruby	regulation of protein ubiquitination	Y
CG18572	rudimentary	dihydroorotase activity	Y
CG5505	scrawny	ubiquitin thiolesterase activity	Y

Table 2.5A (continued)

CG	Protein Name	Function	Conserved in Vertebrates
CG5661	Semaphorin-5c	receptor activity	Y
CG16720	Serotonin receptor 1A	serotonin receptor activity	Y
CG4299	Set	cyclin binding	Y
CG6939	SET domain binding factor	protein phosphatase activity	Y
CG43770	sex lethal	RNA splicing	Y [1]
CG6987	SF2	RNA splicing	Y
CG8582	Sh3beta	unknown	Y
CG18397	short spindle 3	mitotic spindle elongation	Y
CG18076	short stop	microtubule binding	Y
CG12819	slender lobes [2]	nucleolus organization	N
CG5094	small glutamine-rich tetratricopeptide containing protein	binding	Y
CG1391	small optic lobes	proteolysis	Y
CG6369	Smg6	nonsense-mediated decay	Y
CG7528	Smt3 activating enzyme 2	protein sumoylation	Y
CG1982/CG4649	sorbitol dehydrogenase	oxidoreductase activity	Y
CG13570	spaghetti	protein phosphatase activity	Y
CG11451	Spc105-related	mitotic spindle organization	N [3]
CG17286	spindle defective 2	mitotic spindle organization	Y
CG15415	spindly	regulation of metaphase/anaphase transition	Y
CG4602	Srp54	RNA splicing	Y
CG5434	Srp72	7S RNA binding	Y
CG31641	stathmin	microtubule binding	Y
CG17246	Succinate dehydrogenase A	succinate dehydrogenase activity	Y
CG3283	succinate dehydrogenase B	succinate dehydrogenase (ubiquinone) activity	Y
CG8409	Suppressor of variegation 205	regulation of transcription	Y
CG16725	survival motor neuron	RNA binding	Y
CG33094	syndapin	synaptic vesicle endocytosis	Y

Table 2.5A (continued)

CG	Protein Name	Function	Conserved in Vertebrates
CG8439	T-complex Chaperonin 5	mitotic spindle organization	Y
CG8351	Tcp-1eta	mitotic spindle organization	Y
CG2331	TER94	proteolysis	Y
CG1274	thioredoxin peroxidase 2	antioxidant activity	Y
CG9765	transforming acidic coiled-coil protein	microtubule binding	Y
CG8309	Transport and Golgi organization 7	golgi organization	Y
CG1782	ubiquitin activating enzyme 1	ubiquitin activating enzyme activity	Y
CG4347	UGP	UTP:glucose-1-phosphate uridylyltransferase activity	Y
CG12359	Ulp1	proteolysis	N
CG2174	unconventional myosin class XV	intracellular protein transport	Y
CG5014	vap-33-1	neuromuscular junction development	Y
CG11844	vig2	heterochromatin organization	Y
CG10682	vihar	regulation of cell cycle	Y
CG31764	virus-induced RNA 1	defense response to virus	N
CG14542	vps2	protein transport	Y
CG8390	vulcan	cell-cell signaling	Y
CG13176	washout	oogenesis	Y
CG42396	wech	unknown	Y
CG4148	weckle	regulation of transcription	Y
CG5344	whacked	Rab GTPase activator activity	Y
CG4548	XNP	regulation of JNK cascade	Y
CG2985	yolk protein 1	vitellogenesis	N
CG2979	yolk protein 2	vitellogenesis	N
CG11129	yolk protein 3	vitellogenesis	N
CG1372	yolkless	vitellogenesis	N
CG3994	ZnT35C	zinc ion transmembrane transporter activity	Y

Table 2.5A (continued)

CG	Protein Name	Function	Conserved in Vertebrates
CG10011		unknown	Y
CG10083		actin binding	Y
CG10103		unknown	Y
CG10185		unknown	Y
CG10417		protein phosphatase activity	Y
CG10588		proteolysis	Y
CG11188		unknown	Y
CG11236		oxidation reduction	Y
CG11395		unknown	Y
CG11414		protein binding	Y
CG12945		unknown	N
CG13151		DNA binding	N
CG1317		zinc ion binding	Y
CG13349		proteasome assembly	Y
CG13350		DNA binding	Y
CG13373		unknown	Y
CG13630		proteolysis	Y
CG13907		transmembrane transport	Y
CG14184		unknown	Y
CG14309		unknown	N
CG14446		unknown	Y
CG14749		poly(A)+ mRNA export from nucleus	Y
CG15237		unknown	N
CG1597		oligosaccharide metabolic process	Y
CG1646		RNA splicing	Y
CG1647		zinc ion binding	N

Table 2.5A (continued)

CG	Protein Name	Function	Conserved in Vertebrates
CG16903		cyclin-dependent protein kinase regulator activity	Y
CG16952		protein binding	Y
CG1703		transporter activity	Y
CG17261		unknown	N
CG18190		microtubule binding	N
CG18259		nucleotide binding	Y
CG18273		unknown	N
CG18659		unknown	Y
CG1910		unknown	N
CG2091		RNA catabolic process	Y
CG2263		tRNA binding	Y
CG2774		phosphatidylinositol binding	Y
CG2915		proteolysis	Y
CG30101		unknown	Y
CG30115		regulation of Rho protein signal transduction	Y
CG30122		RNA splicing	Y
CG31108		tubulin-tyrosine ligase activity	Y
CG31368		RNA splicing	Y
CG32088		unknown	N
CG32206		unknown	N
CG3223		unknown	N
CG32626		protein binding	Y
CG3368		unknown	N
CG34356		protein kinase activity	N
CG3511		protein folding	Y
CG3590		purine nucleotide metabolic process	Y
CG3632		protein phosphatase activity	Y

Table 2.5A (continued)

CG	Protein Name	Function	Conserved in Vertebrates
CG3689		mRNA cleavage	Y
CG3759		oxidation reduction	N
CG3760		unknown	Y
CG42554		unknown	N
CG42669		unknown	N
CG42748		zinc ion binding	N
CG4434		oxidation reduction	N
CG4857		unknown	N
CG4968		protease	Y
CG5131		KU70 binding	Y
CG5195		protein binding	Y
CG5245		zinc ion binding	Y
CG5315		hormone binding	Y
CG5384		microtubule associated complex	Y
CG5525		mitotic spindle organization	Y
CG5527		proteolysis	N
CG5554		protein disulfide isomerase activity	Y
CG5602		DNA ligase activity	Y
CG5880		zinc ion binding	Y
CG5921		unknown	Y
CG6052		transporter activity	Y
CG6118		mitotic spindle organization	N
CG6129		regulation of transcription	Y
CG6254		zinc ion binding	Y
CG6509		antimicrobial humoral response	Y
CG6540		unknown	Y
CG6751		general RNA polymerase II transcription factor activity	Y

Table 2.5A (continued)

CG	Protein Name	Function	Conserved in Vertebrates
CG6904		glycogen synthase activity	Y
CG7044		unknown	Y
CG7565		chitinase activity	Y
CG7927		unknown	Y
CG7967		unknown	Y
CG7997		carbohydrate metabolic process	Y
CG8060		protein binding	Y
CG8197		protein binding	Y
CG8209		zinc ion binding	Y
CG8223		unknown	Y
CG8909		calcium ion binding	Y
CG9154		methylation	Y
CG9360		oxidation reduction	Y
CG9468		zinc ion binding	Y
CG9512		choline dehydrogenase activity	Y
CG9601		unknown	Y
CG9609		zinc ion binding	Y
CG9667		RNA splicing	Y
CG9705		regulation of transcription	Y
CG9727		regulation of transcription	Y

[1] InParanoid identifies a ortholog in gallus gallus: Ensembl ID ENSGALT00000012486

[2] some peptides also align to CG12592 which is a gene prediction at the junction of a tandemly duplicated region of which the 3' end is highly homolgous to the 3' end of flanking gene slender lobes. (FlyBase)

[3] Schittenhelm et al, 2009 identifies this gene as a homologue of the human protein Blinkin and other eukaryotic Spc105/Spc7/KNL-1/Blinkin protein family members

Table 2.5B CG	Expression	Evidence of Phosphorylation	Method of Identification	Phosphorylated in
CG31196	up in multiple tissues, including ovary	Y^D	2D gel	activated egg
CG17870	up in multiple tissues, not ovary	Y	2D gel	activated egg
CG8947	up in ovary	N	2D gel	activated egg
CG13388	up in multiple tissues, including ovary	Y^D	both	oocyte (2D gel) / activated egg (IMAC)
	expressed everywhere	Y	2D gel	both
CG5712	up in multiple tissues, not ovary	N* (not on tyrosine)	IMAC	oocyte
CG12131	up in multiple tissues, including ovary	Y	2D gel	activated egg
CG1070	up in ovary and larval CNS	Y	IMAC	oocyte
CG9556	up in multiple tissues, including ovary	Y	2D gel	oocyte
CG4260	up in multiple tissues, not ovary	Y^D	IMAC	oocyte
CG1906	NA	Y^D	2D gel	oocyte
CG8308	up in ovary	N	2D gel	oocyte
CG2446	up in multiple tissues, including ovary	Y^D	IMAC	oocyte
CG6631	up in multiple tissues, including ovary	N	IMAC	oocyte
CG1451	up in multiple tissues, including ovary	Y	IMAC	oocyte
CG11154	up in multiple tissues, not ovary	Y	2D gel	oocyte
CG6137	up in ovary	N	IMAC	oocyte
CG6877	up in multiple tissues, including ovary	Y	IMAC	oocyte
CG7926	up in multiple tissues, including ovary	Y	IMAC	oocyte
CG6386	up in ovary and larval CNS	Y^D	IMAC	oocyte
CG13425	up in multiple tissues, not ovary	Y	2D gel	oocyte
CG5529	up in multiple tissues, not ovary	N	IMAC	activated egg
CG3612	up in multiple tissues, not ovary	Y	2D gel	oocyte
CG8276	up in multiple tissues, including ovary	Y	IMAC	oocyte
CG6546	up in ovary and larval CNS	N	2D gel	oocyte
CG10542	up in ovary, brain, and larval CNS	Y	IMAC	activated egg
CG9097	up in multiple tissues, not ovary	Y	IMAC	activated egg
CG42344	NA	Y	IMAC	oocyte

Table 2.5B (continued)

CG	Expression	Evidence of Phosphorylation	Method of Identification	Phosphorylated in
CG31132	up in ovary, brain, and larval CNS	Y	IMAC	oocyte
CG12892	up in ovary	Y^D	IMAC	oocyte
CG11958	expressed everywhere	Y^D	IMAC	oocyte
CG12019	up in multiple tissues, including ovary	Y^D	IMAC	activated egg
CG7597	up in multiple tissues, including ovary	Y	IMAC	activated egg
CG7464	up in multiple tissues, not ovary	N	IMAC	oocyte
CG4145	up in multiple tissues, not ovary	N	IMAC	activated egg
CG33957	up in multiple tissues, not ovary	Y	IMAC	oocyte
CG34389	up in multiple tissues, including ovary	N	IMAC	activated egg
CG11181	up in ovary and testis	Y^D	IMAC	oocyte
CG6081	up in multiple tissues, not ovary	N	IMAC	oocyte
CG6692	up in multiple tissues, not ovary	N	2D gel	activated egg
CG9745	up in multiple tissues, including ovary	Y^D	IMAC	oocyte
CG2207	up in ovary, larval CNS, and larval trachea	Y^D	2D gel	activated egg
CG2175	up in ovary	N	IMAC	oocyte
CG42799	NA	N	IMAC	activated egg
CG1938	up in multiple tissues, including ovary	Y^D	both	oocyte
CG8598	up in ovary and larval salivary gland	Y	IMAC	oocyte
CG4912	up in multiple tissues, including ovary	Y^D	2D gel	activated egg
CG9841	up in multiple tissues, including ovary	N	IMAC	oocyte
CG6542	up in ovary, fat body, and spermatheca	Y	IMAC	oocyte
CG10315	up in multiple tissues, including ovary	Y	IMAC	oocyte
CG32859	up in testis	Y	IMAC	activated egg
CG10840	up in multiple tissues, including ovary	Y^D	IMAC	oocyte
CG5345	up in multiple tissues, including ovary	Y	IMAC	oocyte
CG4930	up in multiple tissues, including ovary	N	2D gel	oocyte

Table 2.5B (continued)

CG	Expression	Evidence of Phosphorylation	Method of Identification	Phosphorylated in
CG16932	up in multiple tissues, including ovary	Y	IMAC	activated egg
CG10837	up in multiple tissues, including ovary	Y^{D}	IMAC	oocyte
CG9946	up in male accessory glands, larval salivary gland and trachea	Y^{D}	2D gel	oocyte
CG9075	up in multiple tissues, not ovary	N	2D gel	oocyte
CG10192	up in multiple tissues, including ovary	N	IMAC	activated egg
CG15573	up in ovary	N	IMAC	oocyte
CG4535	up in multiple tissues, including ovary	Y	2D gel	oocyte
CG9611	up in multiple tissues, not ovary	Y	IMAC	oocyte
CG7004	up in multiple tissues, including ovary	N	IMAC	activated egg
CG11086	up in multiple tissues, not ovary	N	IMAC	oocyte
CG3183	up in ovary and larval CNS	Y^{D}	2D gel	oocyte
CG5272	up in ovary	Y^{D}	IMAC	oocyte
CG5320	up in multiple tissues, not ovary	Y	2D gel	oocyte
CG8893	up in multiple tissues, including ovary	Y^{D}	2D gel	activated egg
CG7254	up in multiple tissues, not ovary	N* (not on threonine)	IMAC	activated egg
CG5520	up in multiple tissues, not ovary	Y	2D gel	activated egg
CG8384	expressed everywhere	Y^{D}	IMAC	oocyte
CG4183	up in ovary and larval tubule	Y^{D}	both	oocyte (2D gel) / both (IMAC)
CG4466	up in multiple tissues, including ovary	Y^{D}	2D gel	oocyte
CG1242	up in ovary and larval salivary gland	Y^{D}	2D gel	both
CG4264	up in multiple tissues, not ovary	Y	2D gel	activated egg
CG4261	up in multiple tissues, including ovary	Y	IMAC	oocyte
CG7269	up in ovary and larval CNS	Y	IMAC	oocyte
CG3373	expressed everywhere	$Y^{D\,4}$	IMAC	oocyte
CG4451	up in multiple tissues, including ovary	N	IMAC	oocyte
CG12749	up in multiple tissues, including ovary	Y	IMAC	oocyte

Table 2.5B (continued)

CG	Expression	Evidence of Phosphorylation	Method of Identification	Phosphorylated in
CG10473	up in multiple tissues, including ovary	Y^{D}	IMAC	oocyte
CG2947	up in multiple tissues, including ovary	Y	IMAC	oocyte
CG6603	up in multiple tissues, including ovary	Y	2D gel	activated egg
CG3504	up in head and eye	Y^{D}	IMAC	oocyte
CG10101	up in testis	N	IMAC	oocyte
CG3654	up in multiple tissues, including ovary	N	IMAC	activated egg
CG1804	NA	Y	IMAC	oocyte
CG6217	up in multiple tissues, not ovary	N	IMAC	activated egg
CG32464	up in multiple tissues, including ovary	Y^{D}	IMAC	oocyte
CG42551	up in multiple tissues, including ovary	Y^{D}	IMAC	oocyte
CG8597	up in multiple tissues, including ovary	Y^{D}	IMAC	oocyte
CG8465	up in multiple tissues, including ovary	Y^{D}	IMAC	oocyte
CG7085	up in multiple tissues, not ovary	Y	2D gel	oocyte
CG5738	up in multiple tissues, including ovary	N	IMAC	oocyte
CG18543	up in ovary	N	IMAC	oocyte
CG7162	up in multiple tissues, including ovary	Y	IMAC	oocyte
CG3697	up in ovary, larval CNS, and larval trachea	Y	IMAC	oocyte
CG42572	up in ovary and testis	Y	IMAC	activated egg
CG4206	up in ovary and larval CNS	Y^{D}	2D gel	activated egg
CG16973	up in multiple tissues, including ovary	Y^{D}	IMAC	oocyte
CG13922	up in multiple tissues, including ovary	N	2D gel	oocyte
CG18124	up in multiple tissues, including ovary	N	IMAC	activated egg
CG32491	up in multiple tissues, including ovary	Y	IMAC	activated egg
CG2050	up in ovary and male accessory glands	$Y^{D\ 5}$	IMAC	oocyte
CG18740	up in multiple tissues, including ovary	Y	IMAC	activated egg
CG1216	up in multiple tissues, including ovary	N* (not on serine)	IMAC	activated egg

Table 2.5B (continued)

CG	Expression	Evidence of Phosphorylation	Method of Identification	Phosphorylated in
CG11156	up in ovary	Y	IMAC	oocyte
CG9311	up in multiple tissues, including ovary	Y	IMAC	activated egg
CG32156	up in multiple tissues, not ovary	Y	IMAC	oocyte
CG3845	up in multiple tissues, including ovary	Y	IMAC	oocyte
CG17704	NA	Y	IMAC	activated egg
CG17255	up in multiple tissues, including ovary	Y^{D}	IMAC	oocyte
CG32190	up in multiple tissues, not ovary	Y	2D gel	activated egg
CG5330	up in multiple tissues, including ovary	Y^{D}	both	both (2D gel) / oocyte (IMAC)
CR9700	NA	N	IMAC	oocyte
CG5581	up in ovary and larval CNS	$Y^{D\,6}$	IMAC	both
CG6824	up in ovary	N	IMAC	oocyte
CG9050		N	2D gel	activated egg
CG33103	up in multiple tissues, not ovary	N	IMAC	activated egg
CG4845	up in multiple tissues, including ovary	N* (not on tyrosine)	IMAC	activated egg
CG3127	up in multiple tissues, not ovary	Y	2D gel	activated egg
CG1721	up in multiple tissues, not ovary	Y	2D gel	oocyte
CG9183	up in ovary	Y^{D}	IMAC	oocyte
CG8169	up in ovary, testis, and larval CNS	Y	IMAC	activated egg
CG4532	up in multiple tissues, including ovary	Y	IMAC	oocyte
CG7913	up in multiple tissues, including ovary	Y^{D}	IMAC	activated egg
CG30342	up in multiple tissues, including ovary	Y	IMAC	oocyte
CG4904	up in multiple tissues, including ovary	Y	IMAC	oocyte
CG1519	up in multiple tissues, including ovary	Y^{D}	IMAC	oocyte
CG10149	up in multiple tissues, not ovary	Y	IMAC	oocyte
CG4720	up in multiple tissues, including ovary	Y	IMAC	oocyte
CG2049	up in multiple tissues, including ovary	Y	IMAC	oocyte
CG17291	up in multiple tissues, not ovary	N	2D gel	activated egg

Table 2.5B (continued)

CG	Expression	Evidence of Phosphorylation	Method of Identification	Phosphorylated in
CG9181	up in multiple tissues, including ovary	Y[D]	IMAC	oocyte
CG7752	up in multiple tissues, not ovary	N	IMAC	oocyte
CG7070	up in multiple tissues, not ovary	Y	2D gel	oocyte
CG5627	up in multiple tissues, not ovary	Y	IMAC	activated egg
CG1799	up in multiple tissues, including ovary	Y[D]	2D gel	oocyte
CG9412	up in multiple tissues, including ovary	Y[D 7]	IMAC	oocyte
CG31293	up in multiple tissues, including ovary	N	2D gel	oocyte
CG9273	up in ovary and larval CNS	Y	2D gel	activated egg
CG40494	up in multiple tissues, including ovary	Y	IMAC	oocyte
CG6811	up in multiple tissues, including ovary	Y[D]	IMAC	oocyte
CG4755	up in multiple tissues, including ovary	Y[D]	IMAC	oocyte
CG9774	up in multiple tissues, including ovary	Y	IMAC	activated egg
CG5371	up in ovary	N	IMAC	activated egg
CG7434	expressed everywhere	Y[D 8]	IMAC	oocyte
CG1821	expressed everywhere	Y[D]	IMAC	activated egg
CG7490	up in multiple tissues, not ovary	Y[D]	2D gel	both
CG4420	up in multiple tissues, including ovary	Y[D]	IMAC	activated egg
CG12559	up in ovary, male accessory glands, and larval tubule	Y[D]	IMAC	oocyte
CG18174	up in multiple tissues, including ovary	Y	2D gel	activated egg
CG10230	up in multiple tissues, including ovary	Y	2D gel	oocyte
CG16916	up in multiple tissues, including ovary	Y	2D gel	oocyte
CG10955	up in ovary and testis	Y[D]	IMAC	oocyte
CG11427	up in multiple tissues, including ovary	Y	IMAC	activated egg
CG18572	up in ovary, larval CNS, and larval salivary gland	Y	IMAC	activated egg
CG5505	up in multiple tissues, including ovary	Y[D]	IMAC	oocyte

Table 2.5B (continued)

CG	Expression	Evidence of Phosphorylation	Method of Identification	Phosphorylated in
CG5661	up in multiple tissues, including ovary	Y	IMAC	oocyte
CG16720	up in multiple tissues, not ovary	Y [9]	IMAC	oocyte
CG4299	up in ovary, larval CNS, and larval salivary gland	Y^D	2D gel	oocyte
CG6939	up in multiple tissues, including ovary	Y	IMAC	oocyte
CG43770	NA	Y	IMAC	oocyte
CG6987	up in multiple tissues, including ovary	Y	IMAC	oocyte
CG8582	up in multiple tissues, including ovary	Y^D	2D gel	oocyte
CG18397	up in multiple tissues, including ovary	Y	IMAC	oocyte
CG18076	up in multiple tissues, not ovary	Y	IMAC	oocyte
CG12819	up in ovary, testis, and larval CNS	Y^D	IMAC	both
CG5094	up in multiple tissues, including ovary	Y	2D gel	activated egg
CG1391	up in multiple tissues, including ovary	N* (not on tyrosine)	IMAC	oocyte
CG6369	up in multiple tissues, including ovary	Y	IMAC	oocyte
CG7528	up in ovary, larval CNS, and larval salivary gland	Y	both	activated egg
CG1982/CG4649	up in multiple tissues, not ovary	Y	IMAC	oocyte
CG13570	up in ovary, testis, and larval CNS	Y	IMAC	oocyte
CG11451	up in ovary and larval CNS	N	IMAC	oocyte
CG17286	up in ovary	N	IMAC	oocyte
CG15415	up in ovary and larval CNS	Y	IMAC	oocyte
CG4602	up in multiple tissues, including ovary	Y	IMAC	oocyte
CG5434	up in multiple tissues, including ovary	Y	IMAC	oocyte
CG31641	up in ovary and larval CNS	Y	IMAC	oocyte
CG17246	up in multiple tissues, not ovary	Y	2D gel	oocyte
CG3283	up in multiple tissues, not ovary	N	IMAC	oocyte
CG8409	up in ovary, larval CNS, and larval trachea	Y^D	IMAC	activated egg
CG16725	up in ovary, testis, and larval CNS	Y	2D gel	activated egg
CG33094	up in multiple tissues, including ovary	Y	IMAC	oocyte

Table 2.5B (continued)

CG	Expression	Evidence of Phosphorylation	Method of Identification	Phosphorylated in
CG8439	up in ovary, larval salivary gland, and larval trachea	N	2D gel	both
CG8351	up in ovary and larval CNS	N	2D gel	both
CG2331	up in multiple tissues, including ovary	Y^{D}	2D gel	activated egg
CG1274	up in multiple tissues, not ovary	N	2D gel	oocyte
CG9765	up in multiple tissues, including ovary	$Y^{D\ 10}$	IMAC	both
CG8309	up in multiple tissues, not ovary	Y	2D gel	activated egg
CG1782	up in multiple tissues, including ovary	Y	IMAC	oocyte
CG4347	up in multiple tissues, not ovary	N	IMAC	oocyte
CG12359	up in ovary and larval CNS	N	IMAC	oocyte
CG2174	up in multiple tissues, not ovary	N	IMAC	oocyte
CG5014	up in multiple tissues, including ovary	Y	IMAC	oocyte
CG11844	up in ovary and male accessory glands	Y	both	oocyte
CG10682	up in ovary and testis	N	2D gel	oocyte
CG31764	up in multiple tissues, not ovary	N	IMAC	activated egg
CG14542	up in multiple tissues, not ovary	Y	IMAC	oocyte
CG8390	up in ovary and testis	Y	IMAC	oocyte
CG13176	up in testis	Y	IMAC	oocyte
CG42396	NA	Y^{D}	IMAC	activated egg
CG4148	up in ovary	Y	IMAC	oocyte
CG5344	up in multiple tissues, including ovary	N* (not on tyrosine)	IMAC	activated egg
CG4548	up in multiple tissues, including ovary	Y	IMAC	oocyte
CG2985	up in eye, heart and adult carcass	Y^{D}	both	both (2D gel) / oocyte (IMAC)
CG2979	up in heart and adult carcass	Y^{D}	2D gel	both
CG11129	up in multiple tissues, not ovary	Y^{D}	2D gel	activated egg
CG1372	up in ovary	Y^{D}	IMAC	oocyte
CG3994	up in larval tubule, tubule, and testis	N	IMAC	oocyte

Table 2.5B (continued)

CG	Expression	Evidence of Phosphorylation	Method of Identification	Phosphorylated in
CG10011	up in multiple tissues, not ovary	Y	IMAC	oocyte
CG10083	up in multiple tissues, including ovary	Y	IMAC	activated egg
CG10103	up in multiple tissues, including ovary	Y^D	IMAC	oocyte
CG10185	up in head	N	IMAC	oocyte
CG10417	up in multiple tissues, including ovary	Y^D	IMAC	activated egg
CG10588	up in testis and male accesory gland	Y	IMAC	oocyte
CG11188	up in ovary	Y	IMAC	oocyte
CG11236	up in multiple tissues, not ovary	N	IMAC	oocyte
CG11395	up in multiple tissues, not ovary	Y^D	IMAC	activated egg
CG11414	up in multiple tissues, including ovary	Y	IMAC	oocyte
CG12945	up in ovary	N	IMAC	oocyte
CG13151	up in multiple tissues, including ovary	N	IMAC	oocyte
CG1317	expressed everywhere	Y	IMAC	oocyte
CG13349	up in multiple tissues, including ovary	Y^D	2D gel	oocyte
CG13350	up in ovary	Y^D	IMAC	oocyte
CG13373	up in ovary	N	IMAC	oocyte
CG13630	up in multiple tissues, including ovary	Y^D	2D gel	activated egg
CG13907	up in multiple tissues, including ovary	N	IMAC	oocyte
CG14184	up in multiple tissues, including ovary	Y^D	IMAC	oocyte
CG14309	NA	Y^D	both	oocyte
CG14446	up in multiple tissues, including ovary	N	IMAC	activated egg
CG14749	up in ovary	Y	IMAC	oocyte
CG15237	up in multiple tissues, including ovary	N	IMAC	oocyte
CG1597	up in multiple tissues, including ovary	N	IMAC	activated egg
CG1646	up in multiple tissues, including ovary	N	IMAC	oocyte
CG1647	up in multiple tissues, including ovary	N	IMAC	oocyte

Table 2.5B (continued)

CG	Expression	Evidence of Phosphorylation	Method of Identification	Phosphorylated in
CG16903	up in multiple tissues, including ovary	Y^D	IMAC	oocyte
CG16952	up in multiple tissues, including ovary	Y	IMAC	oocyte
CG1703	up in multiple tissues, including ovary	Y^D	IMAC	oocyte
CG17261	up in testis	N	IMAC	activated egg
CG18190	up in ovary	N	both	activated egg (2D gel) / oocyte (IMAC)
CG18259	up in ovary and testis	N	IMAC	oocyte
CG18273	up in multiple tissues, not ovary	N	IMAC	both
CG18659	up in multiple tissues, including ovary	Y	IMAC	activated egg
CG1910	up in multiple tissues, including ovary	N	IMAC	oocyte
CG2091	up in ovary and larval CNS	Y^D	IMAC	oocyte
CG2263	up in multiple tissues, including ovary	Y	2D gel	oocyte
CG2774	expressed everywhere	Y	2D gel	activated egg
CG2915	up in multiple tissues, not ovary	N	2D gel	oocyte
CG30101	up in testis and eye	N	IMAC	oocyte
CG30115	up in multiple tissues, not ovary	N	IMAC	oocyte
CG30122	up in multiple tissues, including ovary	Y^D	IMAC	oocyte
CG31108	up in multiple tissues, including ovary	Y	IMAC	oocyte
CG31368	up in multiple tissues, including ovary	N	IMAC	oocyte
CG32088	up in testis and larval fat body	N	IMAC	oocyte
CG32206	up in multiple tissues, not ovary	N	IMAC	oocyte
CG3223	up in multiple tissues, including ovary	N	2D gel	activated egg
CG32626	up in multiple tissues, not ovary	Y	IMAC	activated egg
CG3368	up in multiple tissues, including ovary	N	IMAC	activated egg
CG34356	up in multiple tissues, not ovary	N	IMAC	oocyte
CG3511	up in ovary, brain, and larval CNS	N	IMAC	oocyte
CG3590	up in multiple tissues, including ovary	Y	2D gel	oocyte
CG3632	up in multiple tissues, including ovary	Y^D	IMAC	oocyte

Table 2.5B (continued)

CG	Expression	Evidence of Phosphorylation	Method of Identification	Phosphorylated in
CG3689	up in multiple tissues, including ovary	N	IMAC	oocyte
CG3759	up in multiple tissues, not ovary	N	IMAC	activated egg
CG3760	up in multiple tissues, including ovary	Y^D	IMAC	oocyte
CG42554	up in multiple tissues, including ovary	N	IMAC	oocyte
CG42669	up in eye, midgut, and testis	Y^D	IMAC	oocyte
CG42748	up in multiple tissues, including ovary	N	IMAC	oocyte
CG4434	up in testis	N	IMAC	oocyte
CG4857	up in ovary	N	IMAC	oocyte
CG4968	up in ovary and testis	N	IMAC	activated egg
CG5131	up in ovary	N	IMAC	activated egg
CG5195	up in multiple tissues, not ovary	N	IMAC	activated egg
CG5245	up in ovary	N	IMAC	oocyte
CG5315	up in multiple tissues, not ovary	N	IMAC	oocyte
CG5384	up in multiple tissues, including ovary	Y	2D gel	oocyte
CG5525	up in ovary and larval CNS	N	2D gel	oocyte
CG5527	up in multiple tissues, not ovary	N	IMAC	oocyte
CG5554	up in multiple tissues, including ovary	Y	IMAC	oocyte
CG5602	up in ovary and larval CNS	Y^D	IMAC	oocyte
CG5880	up in ovary, brain, and thoracicoabdominal ganglion	N	2D gel	activated egg
CG5921	up in multiple tissues, not ovary	N	2D gel	activated egg
CG6052	up in testis	Y	IMAC	oocyte
CG6118	NA	N	IMAC	activated egg
CG6129	up in multiple tissues, not ovary	Y	IMAC	oocyte
CG6254	up in multiple tissues, including ovary	Y	IMAC	oocyte
CG6509	up in multiple tissues, including ovary	Y	IMAC	oocyte
CG6540	up in ovary and larval CNS	Y^D	IMAC	oocyte
CG6751	up in multiple tissues, including ovary	Y	2D gel	activated egg

Table 2.5B (continued)

CG	Expression	Evidence of Phosphorylation	Method of Identification	Phosphorylated in
CG6904	up in multiple tissues, including ovary	Y^D	IMAC	both
CG7044	up in multiple tissues, including ovary	N	IMAC	activated egg
CG7565	up in multiple tissues, not ovary	N	IMAC	oocyte
CG7927	up in multiple tissues, not ovary	N	IMAC	activated egg
CG7967	expressed everywhere	N	2D gel	activated egg
CG7997	up in multiple tissues, not ovary	Y	2D gel	oocyte
CG8060	up in multiple tissues, including ovary	Y	IMAC	oocyte
CG8197	up in testis	Y	IMAC	oocyte
CG8209	up in multiple tissues, including ovary	Y^D	both	oocyte
CG8223	up in multiple tissues, including ovary	Y^D	2D gel	activated egg
CG8909	up in multiple tissues, not ovary	Y	IMAC	oocyte
CG9154	up in multiple tissues, including ovary	Y	2D gel	activated egg
CG9360	up in multiple tissues, not ovary	N	IMAC	oocyte
CG9468	up in midgut	N	IMAC	oocyte
CG9512	up in multiple tissues, not ovary	N	IMAC	oocyte
CG9601	up in ovary, larval CNS, and thoracicoabdominal ganglion	Y	IMAC	activated egg
CG9609	up in ovary and brain	Y	IMAC	activated egg
CG9667	up in multiple tissues, including ovary	N	IMAC	activated egg
CG9705	up in multiple tissues, including ovary	Y^D	2D gel	oocyte
CG9727	up in multiple tissues, including ovary	Y	IMAC	oocyte

[4] Theopold et al. (1996) J Biol Chem 271(22):12708-15.
[5] Perrin et al. (1999) J Biol Chem 274(10):6315-23.
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[7] Zhai et al. (2008) J Proteome Res. 7(4):1675-82.
[8] Zhao et al. (2002) Biochem Biophys Res Commun. 298(1):60-6.
[9] Raymond (1991) J Biol Chem. 266(22):14747-53.
[10] Giet et al. (2002) J Cell Biol. 156(3):437-51.

of phosphomodulated proteins in these classes also agrees with a previous report that found proteins phospho-modulated at the time of fertilization in sea urchins in the classes of calcium binding/regulation, metabolism, and protein translation (Roux et al., 2008) and supports the hypothesis that phosphorylation is a key regulator of many of the events of egg activation. Our results also identify proteins whose functions suggest new pathways to examine for roles in or immediately after egg activation. For example, our finding of phospho-regulation of both the SUMO activating enzyme Uba2 and the SUMO-specific protease Ulp1 suggests that sumoylation may be important during this developmental transition. Consistent with this idea, SUMO germline clones are reported to have defects in both patterning of the embryo and in the syncytial mitotic cell cycles (Nie et al., 2009). Additionally, our identification of multiple zinc-binding proteins suggests that zinc-based processes may be important during this developmental transition in *Drosophila*, as was recently proposed for mammals (Kim et al., 2010; Suzuki et al., 2010).

Phospho-regulated proteins include new regulators of egg activation and embryogenesis

The proteins identified in this study represent a focused set of candidates that we can now test genetically for roles in egg activation and early embryogenesis. This includes a number of proteins that would not have been identified through a traditional genetic screen for female steriles, as approximately one-third of these candidates are essential for viability (flybase.org). We can now take advantage of recent advances in RNAi tools that allow successful knockdown of genes (and their protein products) in

the female germline (Ni et al., 2011) and test these candidates for functional roles during or immediately after egg activation.

Verifying that our approach can uncover players in the oocyte-to-embryo transition, approximately 15% of the proteins we identified are known to be important for female fertility, playing roles in oogenesis and embryogenesis (flybase.org) and others are implicated in the meiotic cell cycle. For example, our finding of phospho-modulation of Spd-2 and Spindly suggested roles for these proteins during or immediately after egg activation. Consistent with this, fertilized embryos from *spd-2* mutant mothers fail to enter the first mitosis due to problems in pronuclear migration and fusion (Dix and Raff, 2007). Likewise, Spindly (a mitotic phosphoprotein in humans) is necessary for the metaphase-anaphase transition in both mitosis and meiosis (Barisic et al., 2010; Chan et al., 2009; Griffis et al., 2007; Zhang et al., 2010). As *Drosophila* oocytes are arrested in metaphase prior to egg activation, we hypothesize that the dephosphorylation of Spindly is involved in regulating its association with dynein and the transition to anaphase.

To test our hypothesis that the proteins identified in this study include novel factors required for egg activation or early embryogenesis we performed a pilot study testing 18 of our candidates. In addition to four proteins required for oogenesis, we found that activity of the protein encoded by the *mrityu* gene is required for initiation of embryo development. We expect future extension of this RNAi screen to the remaining proteins we detected as phospho-modulated will reveal additional new essential players in the egg-to-embryo transition. Once we understand the functional role of these proteins, we will then be able to progress to understanding the importance

of their phospho-regulation by creating transgenic lines that mutate the phosphorylation sites (to residues that can not be phosphorylated and/or phospho-mimetic residues).

Since 82.9% of our candidates have homologs in vertebrates, the pathways thus uncovered are likely to be conserved. Indeed, the importance of calcium, and the early signaling through calcineurin in egg activation, are conserved between *Drosophila*, sea urchins, and vertebrates [reviewed in Ducibella and Fissore, 2008 and Horner and Wolfner, 2008; Takeo et al., 2010]. Our results suggest that in addition to these early events, many other pathways controlling egg activation may be conserved across species. Consistent with this idea, two proteins that we find to be dephosphorylated during *Drosophila* egg activation (Cup and eIF-2alpha), are also dephosphorylated in mouse oocytes at the time of fertilization (Alves et al., 2009; Villaescusa et al., 2006). Thus, in addition to identifying the phospho-regulated proteins during *Drosophila* egg activation, the results we reported here will likely help to uncover the molecular mechanisms that regulate the egg-to-embryo transition even in animals such as mammals where such screens would be prohibitive.

2.5 Conclusions

This study took an alternative proteomic approach to find new genes important for the oocyte-to-embryo transition. By identifying proteins that are not only present, but phospho-modulated during this transition, we have produced an enriched source of new candidate genes important for egg activation and early embryogenesis. Comparison of the proteomes of *Drosophila* mature oocytes and unfertilized activated

eggs identified 311 proteins that change in phosphorylation state during this transition. Many of these proteins fall into functional categories such as calcium binding and regulation of translation, which are related to the known events of egg activation. As the 82.9% of these genes are conserved from *Drosophila* to vertebrates, they also represent candidates that may be tested for roles in egg activation and embryogenesis in other species of interest. Using the genetic tools available for *Drosophila*, including new RNAi lines that successfully knockdown genes in the female germline, we have begun testing these genes for their roles in egg activation and the initiation of development. Within the set of genes we tested, we identified one new gene, *mrityu*, which is necessary for embryos to progress past the first mitosis. We expect that continued testing of these candidates will reveal additional genes previously not known to act during egg activation or embryogenesis.

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CHAPTER THREE

PHOSPHO-REGULATION PATHWAYS DURING EGG ACTIVATION IN DROSOPHILA MELANOGASTER[3]

3.1 Introduction

Phosphorylation is an important post-translational modification that can regulate protein activity by a number of different mechanisms. For example, phosphorylation can be required for a protein's enzymatic activity, as is the case for Mitogen-Activated Protein Kinases (MAPKs) (Cargnello and Roux, 2011), or influence protein-protein interactions [e.g. 14-3-3 proteins (Mackintosh, 2004)]. Through these regulatory functions, phosphorylation is known to play a role in diverse biological processes such as the cell cycle, neuronal function, and signal transduction (Marks, 1996). Another process that can be added to this list is egg activation – the cellular events that convert a mature oocyte into a developmentally competent egg.

Several lines of evidence suggest the importance of phosphorylation changes in egg activation. First, two Drosophila phosphoproteins known to act at the egg-to-embryo transition, Young Arrest (YA) and Giant Nuclei (GNU), are dephosphorylated upon egg activation (Renault et al., 2003; Yu et al., 1999). Second, levels of phospho-MAPKs decrease upon egg activation in Drosophila, Xenopus, mice, and some marine invertebrates (Ferrell et al., 1991; Kubiak et al., 1993; Sackton et al., 2007; Sanghera

[3] A modified form of this chapter will be submitted as Krauchunas A.R.*, Sackton K.L.*, and Wolfner M.F., "Phospho-regulation pathways during egg activation in Drosophila melanogaster". K. Sackton performed the Westerns looking at YA and GNU in oocytes and embryos from *sarah*, *cortex*, and *prage* mutants.

et al., 1991; Shibuya et al., 1992). This inactivates the MAPKs, and may affect phosphorylation levels of MAPK protein targets present in the egg. Third, the phosphatase calcineurin and the kinase Ca^{2+}/calmodulin-dependent kinase II (CamKII) are required for egg activation in multiple species (reviewed in Chapter One). Fourth, proteomic screens in Drosophila and sea urchin have observed large-scale changes of the phosphoproteome during fertilization/egg activation (Krauchunas et al., submitted; Roux et al., 2006; Roux et al., 2008). However, much work remains to fully understand how these phosphorylation changes relate to the events of egg activation. We are just beginning to identify the repertoire of proteins that are phospho-modulated, and in most cases we do not yet know the functional significance of the different phosphorylation states of a given protein. Additionally, we do not know what upstream proteins regulate these phosphorylation changes.

In Drosophila, a small number of genes have been identified whose action is required for the events of egg activation. The *sarah* (*sra*) gene encodes the Drosophila calcipressin (or RCAN1), which regulates the phosphatase calcineurin (Horner et al., 2006; Takeo et al., 2010; Takeo et al., 2006). Embryos laid by *sra* mutant mothers arrest in anaphase of meiosis I, and have defects in maternal mRNA polyadenylation and translation (Horner et al., 2006; Takeo et al., 2006). An identical meiotic arrest is also seen in calcineurin germline clones (Takeo et al., 2010; 2012). The *cortex* (*cort*) gene encodes a meiosis-specific Cdc20, which is a regulatory component of the Anaphase Promoting Complex (APC/c) (Chu et al., 2001; Pesin and Orr-Weaver, 2007). The APC/c is an E3 ubiquitin ligase responsible for degrading a number of maternal proteins during the oocyte-to-embryo transition (Pesin and Orr-Weaver,

2008). Embryos laid by *cort* mutant mothers arrest at metaphase of meiosis II (Page and Orr-Weaver, 1996). Similar to *sra* mutants, these embryos have defects in maternal mRNA polyadenylation and translation, as well as defects in mRNA degradation (Lieberfarb et al., 1996; Tadros et al., 2003). The *prage* (prg) gene is also required for mRNA translation and degradation at the time of egg activation (Tadros et al., 2003). While the exact cell cycle arrest point of embryos laid by *prg* mutant mothers is not known, it appears to be during or around the time of meiosis. Finally, the *wispy* gene encodes a cytoplasmic poly-A polymerase that is required for polyadenylation and translation of proteins in the oocyte and embryo (Benoit et al., 2008; Cui et al., 2008).

Although the genes *sra*, *cort*, and *prg* affect multiple aspects of egg activation, one event that is independent of their function is the decrease in phospho-MAPKs (Sackton et al., 2007). This observation led us to investigate whether other phosphorylation changes rely on these egg activation genes. In this study we ask whether the activity of *sra*, *cort*, and *prg* is needed for the phosphorylation state changes of maternal proteins. We used the changes in phosphorylation state of four proteins, GNU, YA, Spindly, and Vap-33-1, as markers of the phospho-regulation that takes place during egg activation. (Since *wispy* has a significant role in oocyte maturation, in addition to its role during egg activation, we did not include it in this analysis).

GNU, YA and Spindly are all dephosphorylated upon egg activation. GNU is required for the assembly of the Pan Gu kinase complex, which is essential for chromosome condensation and the coupling of S phase and mitosis during early

embryo cell cycles (Lee et al., 2003). The Pan Gu complex is also required for translation of Smaug, a protein that regulates maternal mRNA degradation during egg activation (Tadros et al., 2007). YA is a nuclear protein that is necessary for progression through the first embryonic mitosis (Lin and Wolfner, 1991; Sackton et al., 2009). For YA, dephosphorylation is hypothesized to allow it to disassociate from cytosolic binding partners, permitting it to enter the nucleus where it can then function (Yu et al., 2002). Spindly is another cell cycle regulator previously shown to act during mitosis and meiosis (Barisic et al., 2010; Griffis et al., 2007; Zhang et al., 2010). In mitotic cells Spindly associates with the Rod-Zw10-Zwilch complex and is necessary for the metaphase-to-anaphase transition (Barisic et al., 2010; Chan et al., 2009; Griffis et al., 2007). We hypothesize that Spindly may play the same role during meiosis, and that dephosphorylation of Spindly is important for the release from the metaphase arrest of mature oocytes. In contrast to the other three proteins, Vap-33-1 is phosphorylated during egg activation and its function in the oocyte is currently unknown (Krauchunas et al., submitted).

We find that *sra* and *cort* contribute to the phospho-modulation of all four proteins tested. We also find that degradation of Cort [a process dependent on the APC/c (Pesin and Orr-Weaver, 2007)] fails to occur in *sra* mutants. In contrast, *prg* is only required for the dephosphorylation of YA and Spindly. Thus, we propose that *sra* and *cort* work in a common pathway to regulate the phosphorylation state of many proteins during egg activation, while *prg* acts downstream or in a parallel pathway to regulate a smaller subset of proteins.

3.2 Materials and Methods

Flies: *Drosophila melanogaster* stocks were raised on standard yeast-glucose-agar medium at room temperature on a 12:12 light:dark cycle. To make *sarah* hemizygotes (Horner *et al.*, 2006), sra^{687}/TM3 (Fbal0175443) were crossed to *Df(3R)sbd45, mwh* ^{1} e^{1}/TM6 (FBst0003678) or sra^{A426}/ TM3 (FBal0194825) were crossed to *Df(3R)sbd45, mwh* ^{1} e^{1}/TM3. To make *cortex* hemizygotes (Chu *et al*., 2001), $cort^{QW55}$ $cn^{1}bw^{1}$/CyO, *l(2)DTS513* ^{1} (Fbst0004974) were crossed to *Df(2L)BSC9,* w^{+mC}/SM6a (FBst0006454). *Prage* mutants (Tadros *et al.*, 2003) were prg^{32}/prg^{32} homozygotes obtained from the prg^{32}/FM6 stock. CanB2 germline clones (Takeo *et al*., 2010) were produced by crossing *w;* $P\{FRT\}^{2R\text{-}G13}CanB2^{EP(2)0774}$/CyO *Cy* females with *P{hsFLP}12, y w/Y;* $P\{FRT\}^{2R\text{-}G13}P\{ovo^{D1}\}^{2R}$/CyO *Cy* males. Larvae, at approximately the third instar stage, were heat shocked at 37^{o}C for 2 hours. The effects of constitutively active calcineurin (CnA^{act}) in the germline were tested by expressing UASp-Pp2B-14D^{act} driven by nanos-GAL4 (Takeo et al., 2006). In all cases, balancer siblings were used as controls.

Oocytes, embryos, and protein extraction: Ovaries or stage 14 mature oocytes were obtained from 3-5 day old wild-type virgin females that had been reared on heavily-yeasted food. Dissection was performed in Isolation Buffer, a hypertonic solution that does not activate eggs (Page and Orr-Weaver, 1997). Oocytes were dissected in one hour blocks of time, then flash-frozen in liquid nitrogen.

To obtain embryos, newly eclosed virgin females were aged on yeasted vials for 3-8 days and then mated to wild-type males (OregonR P2; Allis *et al*., 1977). Mated females were allowed to deposit eggs onto petri plates containing grape juice-agar for

0-30 minute, 0-1 hour, or 0-2 hour periods. [No differences were seen in Western blot results for 0-30 minute, 0-1 hour, or 0-2 hour samples. All phosphorylation changes were examined at least once with 0-30 minute samples. Due to the technical difficulties of collecting enough material during 0-30 minute collections, additional biological replicates were sometimes run with 0-1 hour or 0-2 hour samples. Degradation of Cort was examined in either 0-1 hour or 0-2 hour samples.] Eggs were washed off the plates in Egg Wash (Karr and Alberts, 1986), dechorionated in a 50% sodium hypochlorite solution for 2 minutes, and flash frozen in liquid nitrogen. Samples of 40-100 mature oocytes or activated eggs were homogenized in protease inhibiting homogenization buffer (PIHB; Monsma *et al*., 1988) with the addition of two phosphatase inhibitors (20 mM NaF and 10 mM β-glycerophosphate) or in Extraction Buffer (10 mM Tris pH 7.5; 20 mM NaF, 2 mM EGTA, 10 mM DTT, 400 nM okadaic acid, and 2% SDS), followed by the addition of an equal amount of SDS sample buffer. Samples were boiled for 5 minutes and stored at –80^{o}C until use. *Immunoblotting*: Proteins were separated on polyacrylamide SDS gels and subjected to Western blotting analysis as previously described (Sackton *et al*., 2007). Acrylamide percentage varied based on the protein of interest: 7.5% for YA and Spindly, 12% for GNU, 10.6% for Vap-33-1 and Cortex, and 5% for Cdc27. Gels for Vap-33-1 also contained 25 µM Phos-tag and gels for Cdc27 contained 3.5 µM Phos-tag (Wako Pure Chemical Industries, Ltd., Richmond, VA) (Kinoshita *et al*., 2006). Primary antibodies were used at the following dilutions in 1% milk: rabbit anti-YA, 1:1000 (Liu *et al*., 1995); guinea pig anti-GNU, 1:5000 (gift of T. Orr-Weaver; Lee *et al*., 2003); guinea pig anti-Vap-33-1, 1:10,000 (gift of H. Bellen; Pennetta *et al*.,

2002); rabbit anti-Spindly, 1:1000 (gift of R. Vale; Griffis *et al*., 2007); guinea pig anti-Cort, 1:2000 (gift of T. Orr-Weaver; Pesin and Orr-Weaver, 2007); rabbit anti-Cdc27, 1:500 (gift of J. Raff; Huang and Raff, 1999). HRP-conjugated secondary antibodies were used at a 1:2000 dilution and visualized with the ECL Plus Western Blotting Detection system (GE Healthcare, Piscataway, NJ).

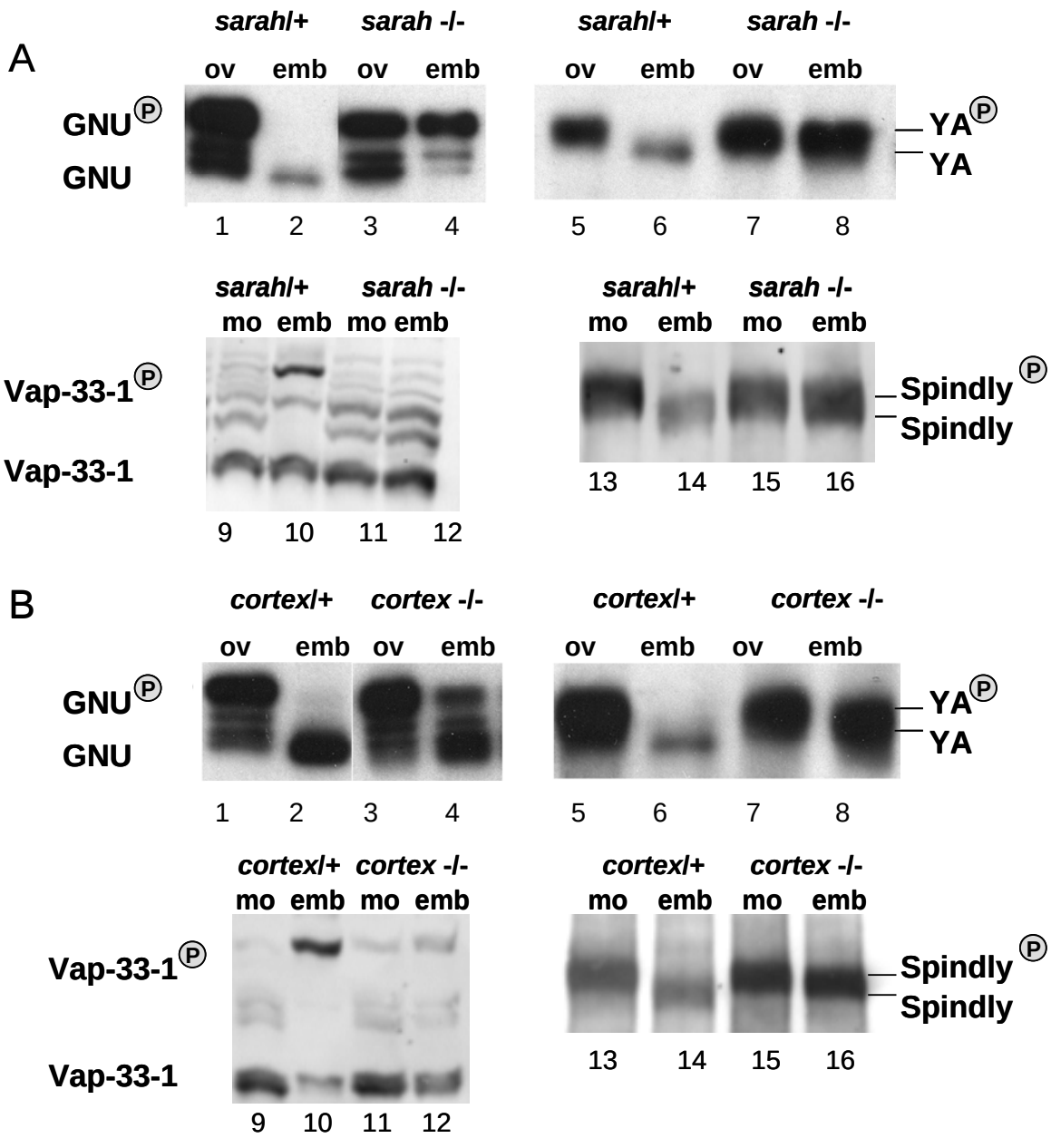
3.3 Results and Discussion

sarah and calcineurin regulate the phosphorylation state of multiple proteins during egg activation

We took advantage of the fact that different phosphorylation states can have different electrophoretic mobilities in a gel; a more highly phosphorylated form of a protein will often have a slower mobility than a less phosphorylated form of the same protein. When egg activation occurs properly, GNU, YA, Vap-33-1, and Spindly all have different electrophoretic mobilities in embryos (or unfertilized activated eggs) as compared to what is seen in mature (stage 14) oocytes. These gel mobility differences are abolished when samples are treated with a phosphatase, showing that the slower migrating forms of these proteins are phosphorylated (Yu *et al*., 1999; Renault *et al*., 2003; Krauchunas *et al*., submitted). Thus, we compared the electrophoretic mobilities of these four proteins in mature oocytes and embryos from *sra* mutants to determine if they underwent the typical changes that are observed upon fertilization. We find that the phosphorylation states of all four proteins are regulated by *sra*.

GNU, YA, Vap-33-1, and Spindly all show normal mobility in oocytes of *sra* mutants (Figure 3.1A, compare lanes 1 and 3, 5 and 7, 9 and 11, 13 and 15), but in all

Figure 3.1 *sarah* and *cortex* function in the phospho-modulation of GNU, YA, Vap-33-1, and Spindly. Western blots of GNU, YA, Vap-33-1 and Spindly in ovaries (ov) or mature oocytes (mo) and embryos (emb) from heterozygotes (controls) and hemizygous mutants. For each protein examined, equal amounts of protein were loaded into each lane based on the number of oocytes and embryos, or micrograms of protein. (A) GNU, YA, Spindly and Vap-33-1 have normal mobilities in oocytes of *sra* mutants as compared to controls. In *sra* embryos, all four proteins fail to shift to the mobilities observed in control embryos. The mobilities of GNU and Vap-33-1 are indistinguishable from oocytes, while YA and Spindly run at intermediate mobilities between control oocytes and embryos. (B) GNU, YA, Spindly and Vap-33-1 have normal mobilities in oocytes of *cort* mutants as compared to controls. In *cort* embryos, all four proteins run at mobilities that are more similar to oocytes than control embryos. A significant fraction of GNU protein remains phosphorylated in *cort* embryos and YA and Spindly run at intermediate mobilities between control oocytes and embryos. The oocyte-specific band of Vap-33-1 can be seen in *cort* embryos, though there may also be a slight increase in the fully phosphorylated form of the protein. Western blots shown are representative of 3 or more independent replicates.


A
sarah/+
sarah -/-
ov
emb
GNU(P)
GNU
YA(P)
YA
1 2 3 4 5 6 7 8
mo
Vap-33-1(P)
Vap-33-1
Spindly(P)
Spindly
9 10 11 12 13 14 15 16
B
cortex/+
cortex -/-

cases lack of *sra* activity prevents the normal shift in gel mobility upon egg activation (Figure 3.1A, compare lanes 2 and 4, 6 and 8, 10 and 12, 14 and 16). GNU mobility in embryos laid by *sra* mutant mothers (hereafter referred to as "*sra* embryos") is comparable to that seen in mature oocytes, rather than the faster gel mobility observed in control embryos (Figure 3.1A, compare lanes 2 and 4). Similarly, the gel mobility of Vap-33-1 in *sra* embryos (Figure 3.1A, lane 12) is indistinguishable from the mobility observed in mature oocytes (Figure 3.1A, lanes 9 and 11). This is in contrast to the mobility of Vap-33-1 in heterozygous control embryos where majority of the protein is present in the slowest mobility band and the band with an intermediate mobility is no longer observed (Figure 3.1A, lane 10). The gel mobilities of YA and Spindly proteins in *sra* embryos are intermediate between their mobilities in mature oocytes and their mobilities in control embryos (Figure 3.1A, compare lane 8 with lanes 6 and 7, lane 16 with lanes 14 and 15). This intermediate mobility is consistent with these proteins being phosphorylated at multiple sites (Yu *et al*., 1999; Yu *et al*., 2002; Griffis et al., 2007; Barisic *et al*., 2010), and suggests that dephosphorylation of some, but not all, of those sites require *sra*. Thus, YA and Spindly are able to be partially, but not fully, dephosphorylated in the absence of Sra.

It has been proposed that the primary role of *sra* is to positively regulate calcineurin (Horner *et al*., 2006; Takeo *et al*., 2010; Takeo *et al*., 2012). If this is true, then loss of calcineurin function in the oocyte should lead to the same phenotypes as observed in *sra* mutants. This has already been shown for the meiotic arrest caused by loss of Sra or calcineurin function in the oocyte (Takeo *et al*., 2010). Our results show that GNU, Spindly, and Vap-33-1 have the same phosphorylation defects in

embryos lacking calcineurin (germline clones) as are seen in *sra* embryos (Figure 3.2). Preliminary results suggest that YA also has the same defect in embryos lacking calcineurin as is seen in *sra* embryos (results not shown.) Without calcineurin activity, these proteins fail to change in phosphorylation state upon egg activation.

Since calcineurin activity is required for the phosphorylation changes of GNU, YA, Spindly, and Vap-33-1, we tested whether expression of constitutively active calcineurin (CnA^{act}) was sufficient to induce any of these phosphorylation changes in the mature oocyte. When CnA^{act} is expressed in the female germline, YA is either partially or fully dephosphorylated in mature oocytes (Figure 3.3, lanes 3 and 7). This suggests that either the activity of calcineurin is sufficient to dephosphorylate YA, or mis-expression of calcineurin during oogenesis prevents YA from being properly phosphorylated.

In contrast, the phosphorylation states of GNU, Spindly, and Vap-33-1 were unaffected by the expression of CnA^{act} in mature oocytes and embryos (Figure 3.3 and data not shown). The mobilities of these proteins are the same in mature oocytes expressing CnA^{act} and control oocytes (Figure 3.3, compare lanes 9 and 11, 13 and 15). The phosphorylation changes of GNU, Spindly, and Vap-33-1 that take place at egg activation were also unaffected by expression of CnA^{act} (Figure 3.3, compare lanes 10 and 12, 14 and 16). One explanation why active calcineurin does not induce phosphorylation changes for GNU, Spindly, and Vap-33-1 is that other factors, active in the embryo, are not yet active in the mature oocyte. If calcineurin requires other proteins to bind or recruit its targets then these additional factors must also be activated for calcineurin to exert its function. If these proteins are held in an inactive

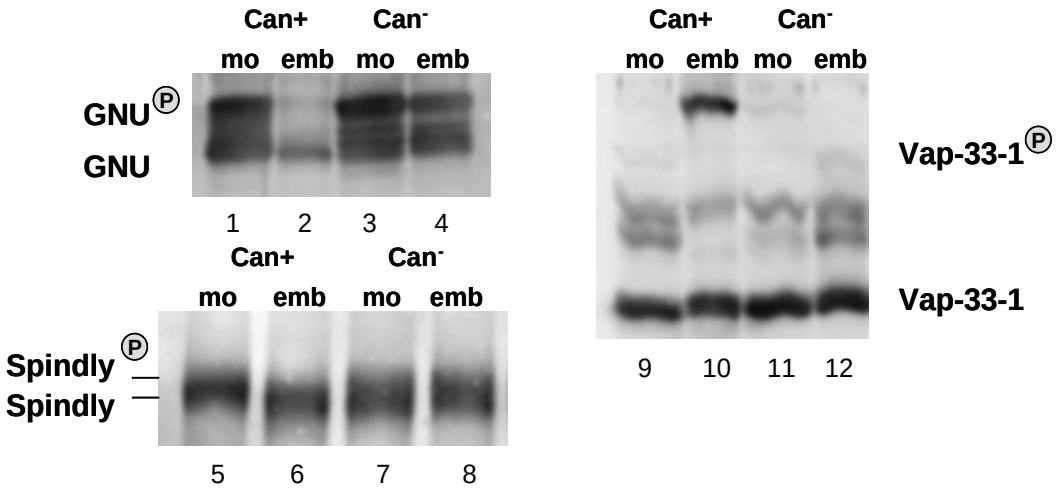


Figure 3.2 Lack of calcineurin activity in oocytes and embryos causes the same mobility phenotypes as seen in *sarah* mutants. No differences can be seen in the mobilities of GNU, Vap-33-1, and Spindly in mature oocytes (mo) lacking calcineurin as compared to controls. In embryos (emb) lacking calcineurin activity, GNU, Vap-33-1, and Spindly have the same mobilities as are seen in oocytes, not control embryos. Western blots shown are representative of two biological replicates.

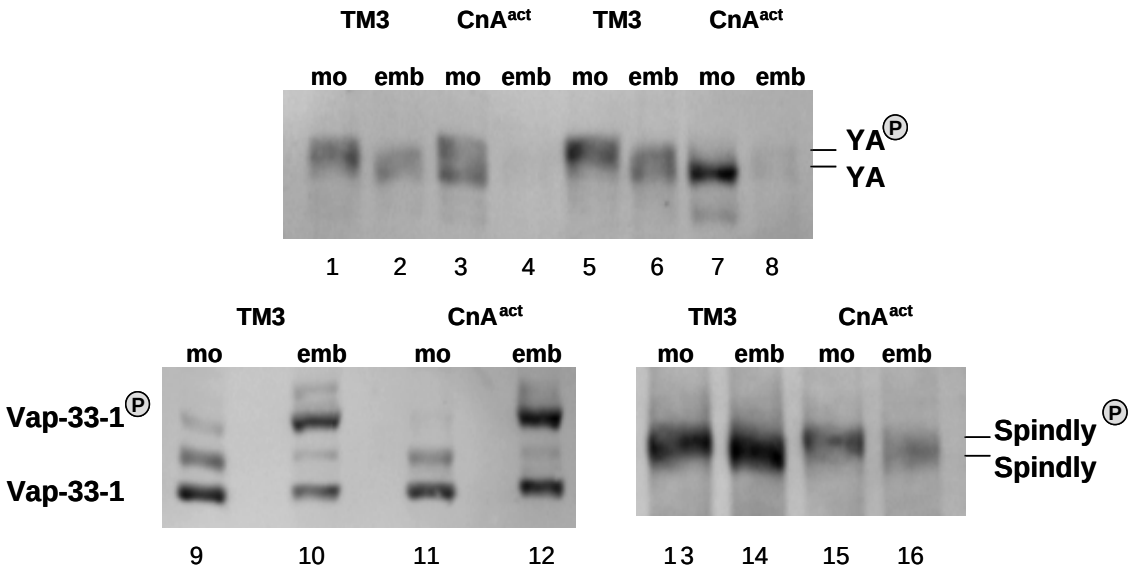


Figure 3.3 YA is not fully phosphorylated in oocytes (mo) expressing constitutively active calcineurin (CnA^{act}) and appears to be degraded in embryos (emb) expressing CnA^{act}. The mobilities of Vap-33-1 and Spindly are normal in oocytes and embryos expressing CnA^{act}. TM3 are balancer sibling controls.

state in the mature oocyte, then we may fail to see an effect of constitutively active calcineurin in the oocyte. Sra is phosphorylated upon egg activation, and this phosphorylation is required for its activity (Takeo et al., 2010; Takeo et al., 2012). Thus, if Sarah (or additional unidentified regulators) is required for calcineurin to recognize or bind its targets, and Sarah is not active in the mature oocyte, then even constitutively active calcineurin may not be able to dephosphorylate its targets until egg activation.

cortex is required for the phosphorylation state changes of GNU, YA, Spindly and Vap-33-1 upon egg activation

As *cortex* (*cort*) is another gene whose mutants show defects in multiple egg activation events (Pesin and Orr-Weaver, 2008; Tadros *et al*., 2003; Page and Orr-Weaver, 1996; Lieberfarb *et al*., 1996), we examined whether *cortex* is also required for any of the phosphorylation changes that take place at egg activation. We find that embryos laid by *cort* mutant mothers ("*cort* embryos") have defects in the dephosphorylation of GNU, YA, and Spindly, as well as in the phosphorylation of Vap-33-1 (Figure 3.1B). Similar to what is seen in *sra* embryos, YA and Spindly in *cort* embryos have mobilities in between the mobilities of either oocytes or control embryos, indicating that partial dephosphorylation takes place (Figure 3.1B, compare lane 8 with lanes 6 and 7, lane 16 with lanes 14 and 15). Likewise, *cort* embryos retain a proportion of GNU in its phosphorylated form. But unlike in *sra* embryos, a significant fraction of GNU protein is still dephosphorylated in the absence of Cort (Figure 3.1B, lane 4). In addition, *cort* embryos retain the mature oocyte specific

phospho-state of Vap-33-1, but also show a small increase in the slowest mobility form of the protein (Figure 3.1B). Thus, the phosphorylation defects in *cort* embryos are less severe than those seen in *sra* or calcineurin mutants.

The finding that GNU, YA, Spindly, and Vap-33-1 all require both *sra* and *cort* for their phosphorylation changes upon egg activation suggests that *sra* and *cort* work in a common pathway to regulate the phosphorylation of these, and possibly other, proteins. As the defects in *cort* embryos are not as complete as in *sra* embryos, we propose that *cort* acts downstream of *sra*. However, it is important to note that the *cort* mutation is a single point mutation and though it has been proposed to be a functional null, it is possible that this allele retains some function (Chu et al., 2001; Pesin and Orr-Weaver, 2007). In addition, the canonical Cdc20, *fizzy*, is also present in the Drosophila oocyte and embryo (Swan and Schupbach, 2007). Both of these factor could contribute to our finding that the defects in phosphorylation changes are less severe in *cort* embryos than in *sra* embryos.

If *cort* does act downstream of *sra*, one hypothesis is that the defects observed in *sra* embryos are then due to mis-regulation of *cort* and at least one other protein/pathway to result in the complete *sra* phenotype. This scenario would also imply none of these four proteins (GNU, YA, Spindly, or Vap-33-1) are direct targets of calcineurin. Alternatively, it is possible that calcineurin directly dephosphorylates GNU, YA, or Spindly and that *cort* (through the APC/c) is necessary to inhibit the kinase(s) that phosphorylate these proteins in the oocyte. Both GNU and YA contain predicted MAPK sites (Renault et al., 2003; Zhang et al., 2004; Yu et al., 1999; Yu et al., 2002), while Spindly contains seven consensus Cdk1 sites (Griffis et al., 2007).

Since MAPK activity decreases normally in *sra* and *cort* embryos (Sackton *et al*., 2007), there are either additional kinases that phosphorylate these proteins or activation of at least one phosphatase is required for the dephosphorylation of GNU and YA.

sarah **is required for Cort degradation after egg activation**

Our hypothesis that *sra* acts upstream of *cort* is supported by the fact that in Xenopus, Cdc20 is dephosphorylated during egg activation and this dephosphorylation requires calcineurin (Mochida and Hunt, 2007). In Drosophila, Cort (a meiosis-specific Cdc20) is degraded by the APC/c upon egg activation (Pesin and Orr-Weaver, 2007). We looked at whether Cort is degraded in *sra* embryos. We find that *sra* is required for Cort degradation after egg activation (Figure 3.4). In control embryos, Cort is present at less than 50% of the levels observed in mature oocytes. In contrast, in *sra* embryos the amount of Cort protein present is 80% of the amount observed in *sra* mature oocytes (Figure 3.4B). These data support our model in which *cort* acts downstream of *sra* in a common pathway, leading to the overlapping requirements of *sra* and *cort* for the phospho-regulation of GNU, YA, Spindly and Vap-33-1. Since the degradation of Cort is dependent on the function of the APC/c, this could mean that the APC/c is not fully functional in the absence of Sra. Sra could regulate APC/c activity through Cort or other subunits of the complex. Alternatively, Sra could regulate Cort stability by targeting it for degradation.

In Xenopus, another subunit of the APC/c, Apc3, is also dephosphorylated in a calcineurin-dependent manner upon egg activation (Mochida and Hunt, 2007). To test

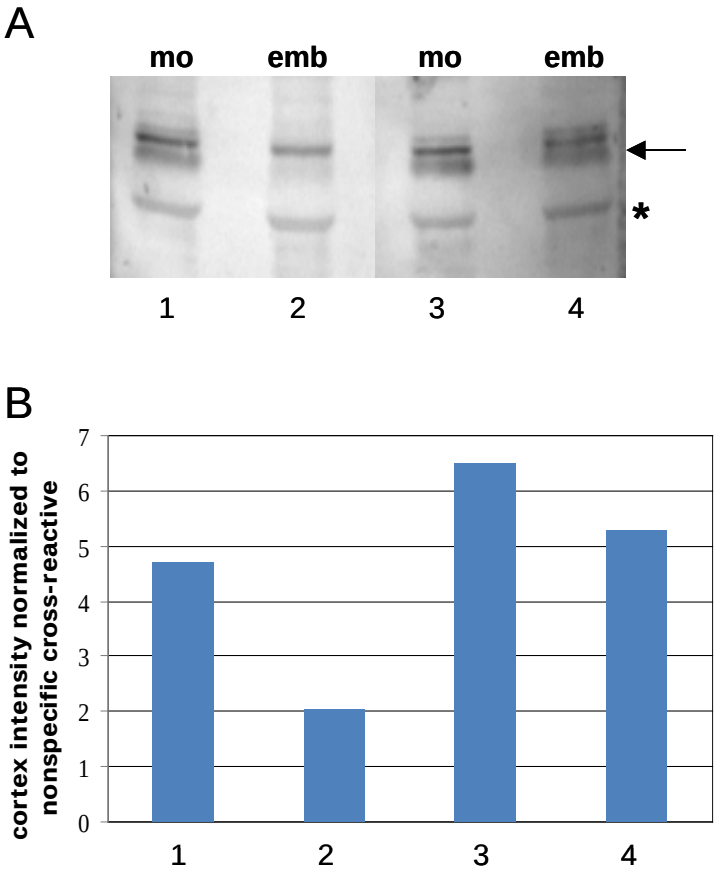


Figure 3.4 Cortex is not degraded in *sarah* embryos. (A) Western blot showing Cort (arrow) in mature oocytes (mo) and embryos (emb) from *sra* heterozygotes (controls) and *sra* hemizygous mutants. Equal amounts of protein were loaded into each lane. Levels of Cort appear similar between control and *sra* oocytes, but remain high in *sra* embryos compared to controls. Non-specific cross-reactive is marked by *. (B) Levels of Cort were quantified by normalizing to the *cross-reactive. Numbers on x-axis correspond to the lane numbers in (A).

whether the same regulation occurs in Drosophila, we first tested whether the Drosophila ortholog of Apc3, Cdc27, is similarly dephosphorylated during egg activation. When run on a polyacrylamide gel containing Phos-tag, which slows the mobility of phosphorylated proteins, we see a slower mobility band of Cdc27 only in mature oocytes (Figure 3.5A). This indicates that, as in Xenopus, egg activation triggers the dephosphorylation of Cdc27 in Drosophila embryos. We then examined whether the dephosphorylation of Cdc27 occurs in *sra* embryos. In contrast to the situation for Xenopus Apc3, we find that *sra* is not required for Cdc27 dephosphorylation in embryos (Figure 3.5B). Therefore, while regulation of Cdc20 by calcineurin appears to be conserved between Xenopus and Drosophila, other specific targets of calcineurin may not be.

prage is only required for a subset of phosphorylation changes at egg activation

We next asked whether *prg* acts in the same pathway as *sra* and *cort* to regulate the phosphorylation states of GNU, YA, Spindly, and Vap-33-1 during egg activation. We find that YA and Spindly have the same mobilities in embryos laid by *prg* mutant mothers ("*prg* embryos"), as is observed in *prg* mature oocytes (Figure 3.6, compare lanes 7 and 8, 15 and 16), though for YA this is a slightly lower mobility than seen in control oocytes (Figure 3.6, compare lanes 15 and 16 with lane 13). Thus, like *sra* and *cort*, *prg* is required for dephosphorylation of YA and Spindly. In addition, the failure to dephosphorylate both proteins is more complete than in either *sra* or *cort* embryos where intermediate mobilities were observed. In contrast, *prg* does not regulate the phosphorylation states of GNU and Vap-33-1, as the mobilities

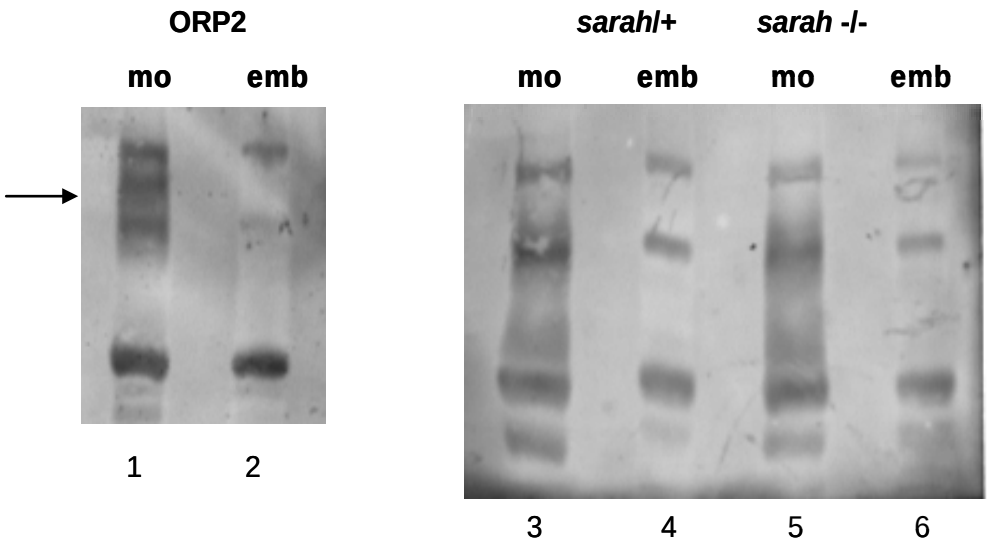


Figure 3.5 Cdc27 is dephosphorylated upon egg activation, but this is independent of *sarah*. (A) Cdc27 has a higher mobility band in ORP2 (wild-type) mature oocytes (lane 1, arrow) that is not present in ORP2 embryos (lane 2). (B) No difference in Cdc27 mobility is observed between mature oocytes from *sra* mutants or controls (lanes 3 and 5) or between *sra* embryos and control embryos (lanes 4 and 6).

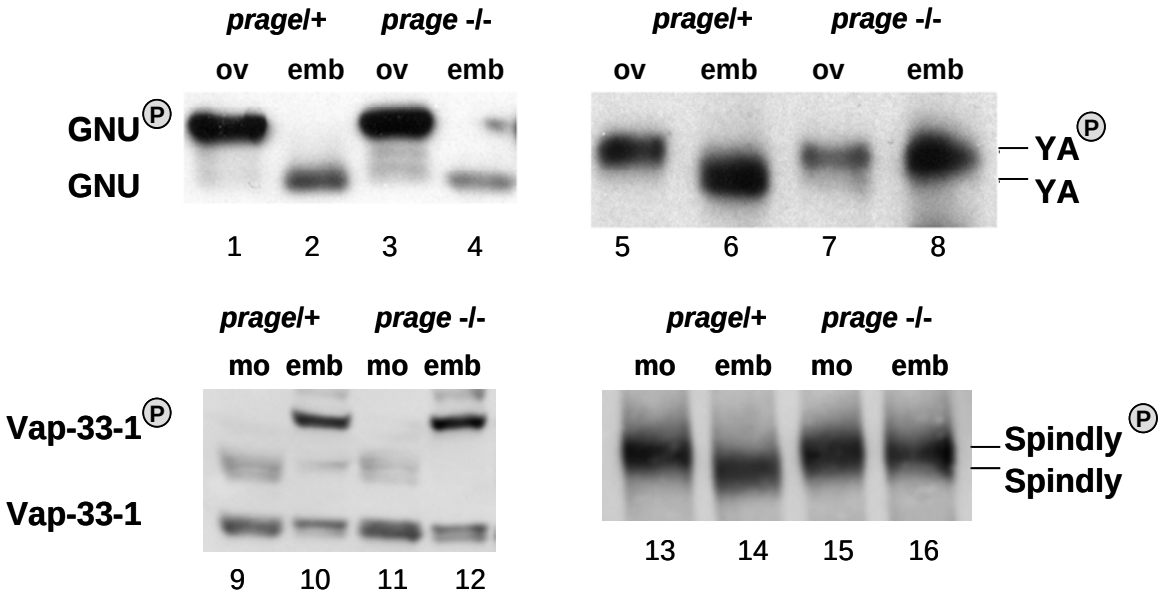


Figure 3.6 ***prage*** **is required for YA and Spindly dephosphorylation, but not phospho-modulation of GNU or Vap-33-1.** For each protein examined equivalent amounts of protein were loaded into each lane. No differences are seen in GNU or Vap-33-1 mobilities between *prg* mutant and control oocytes, or between *prg* embryos and control embryos. The mobilities of YA and Spindly in *prg* embryos are the same as those seen in *prg* oocytes. These proteins fail to shift to the mobilities seen in control embryos.

of GNU and Vap-33-1 in *prg* embryos are indistinguishable from those observed in control embryos (Figure 3.6, compare lanes 2 and 4, 10 and 12). These results show that there are at least two subsets of phosphorylation changes that are regulated during egg activation, *prg*-dependent and *prg*-independent. These findings also suggest that *prg* works downstream of, or in parallel to the *sra/cort* pathway to regulate the *prg*-dependent subset of phosphorylated proteins. If *prg* were upstream of *sra* and *cortex*, we would expect that *prg* would also be required for the phosphorylation changes of GNU and Vap-33-1 that take place at egg activation. Consistent with *prg* being downstream or parallel with *sra* and *cort*, we find that Cort is degraded in *prg* embryos (Figure 3.7). In control embryos, Cort is present at approximately 30% the levels observed in mature oocytes and in *prg* embryos we observed only 22% of the levels observed in mature oocytes (Figure 3.7B).

3.4 Conclusions

Large-scale phosphorylation changes have been observed at the time of egg activation/fertilization in Drosophila (Krauchunas *et al*., submitted) and sea urchins (Roux *et al*., 2006; 2008). In Xenopus and mouse, phosphorylation regulators such as Ca^{2+}/calmodulin-dependent kinase II (CaMKII) and calcineurin are critical effectors of egg activation events (reviewed in Chapter One). However, we still have a limited understanding of which genes and enzymes regulate of the phosphorylation changes at egg activation. How many proteins are direct targets of CaMKII or calcineurin and how many phosphorylation changes are indirect effects due to not yet identified phosphatases and kinases? Do the known egg activation genes regulate unique

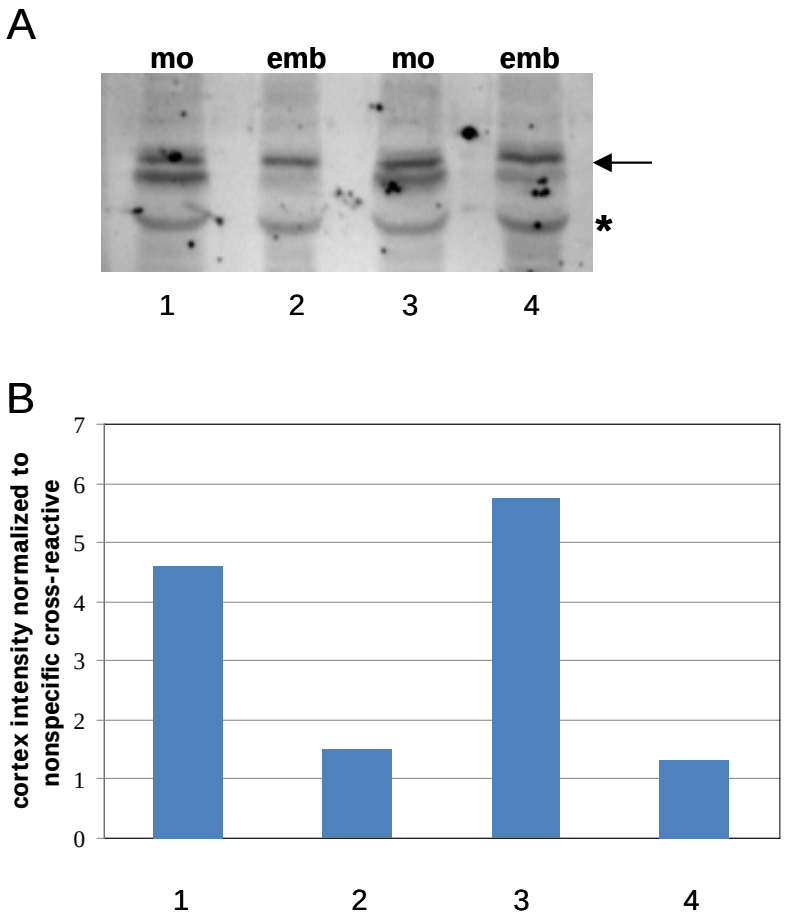


Figure 3.7 Cortex degradation is independent of *prage*. (A) Western blot showing Cort (arrow) in mature oocytes and embryos from *prg* heterozygotes (controls) and *prg* hemizygous mutants. Equal amounts of protein were loaded into each lane. Levels of Cort appear similar between control and *prg* oocytes, and between *prg* and control embryos. Non-specific cross-reactive is marked by *. (B) Levels of Cort were quantified by normalizing to the *cross-reactive. Numbers on the x-axis correspond to the lane numbers in (A).

downstream events or do they work in common pathways to regulate overlapping groups of proteins? What are the functional consequences of these phosphorylation changes?

We have begun to answer these types of questions using the Drosophila model system. We examined the role of three egg activation genes, *sra*, *cort*, and *prg*, in regulating the phosphorylation state of four proteins: GNU, YA, Spindly, and Vap-33-1. We find that *sra* is required for all four of these proteins to change in phosphorylation state upon egg activation. Since we observe an identical phenotype in calcineurin germline clones, the effects seen in *sra* embryos are most likely due to the misregulation of calcineurin.

The fact that GNU fails to be dephosphorylated in *sra* mutants begins to link phosphorylation changes with functional consequences. GNU acts within the PNG kinase complex which is necessary for the translation of Smaug upon egg activation. It has been shown previously that Smaug is not translated in *sra* embryos (Cui et al., 2008), however no mechanism for how *sra* affects Smaug translation has been proposed. If GNU dephosphorylation is required for its activity, our finding that GNU is not dephosphorylated in *sra* embryos provides one possible explanation for why Smaug translation requires *sra*.

We find that *cort* also regulates the phosphorylation state of GNU, YA, Spindly, and Vap-33-1, though *cort* mutants show a less severe phenotype in this trait than do *sra* embryos. In contrast, *prg* only regulates a subset of these phosphorylation changes; YA and Spindly fail to be dephosphorylated in *prg* embryos, but GNU and Vap-33-1 are unaffected by a lack of *prg*. Finally, we show that the degradation of

Cort requires *sra*, but is independent of *prg*. From these data we propose a model in which *sra* and calcineurin act upstream of *cort* (and the APC/c) to regulate the phosphorylation state of multiple proteins, while *prg* acts downstream or in parallel as an additional regulator of only a subset of the *sra/cort* targets (Figure 3.8). Thus, we have shown a link between known egg activation genes and phospho-regulation during egg activation, and have begun to place these genes into a common pathway. Additional work will be necessary to clarify where *prg* acts in relation to this pathway. At present we are unable to observe the developmental arrest point of *prg* embryos and we have been unsuccessful at developing an antibody that recognizes Prage. These tools will be important to establish if and how Prage is affected by *sra* or *cortex*. Determining the targets of *prg* will begin to address the mechanisms by which *prg* can affect the phosphorylation state of YA and Spindly and may provide additional avenues to test the relationship between *prg*, and the *sra/cortex* pathway.

Changes in the phosphorylation state of many proteins at once can rapidly alter molecular and cellular properties and evidence suggests that phosphorylation changes are a key aspect of egg activation. Therefore, it is important that we figure out the pathways that lead to the global phosphorylation changes taking place at this time (Krauchunas *et al*., submitted). We have shown that the conserved proteins, calcineurin and Cdc20, are upstream of multiple phosphorylation events during egg activation. It is likely through these phosphorylation changes that these egg activation regulators are exerting their effects.

By understanding how these proteins work together to alter the

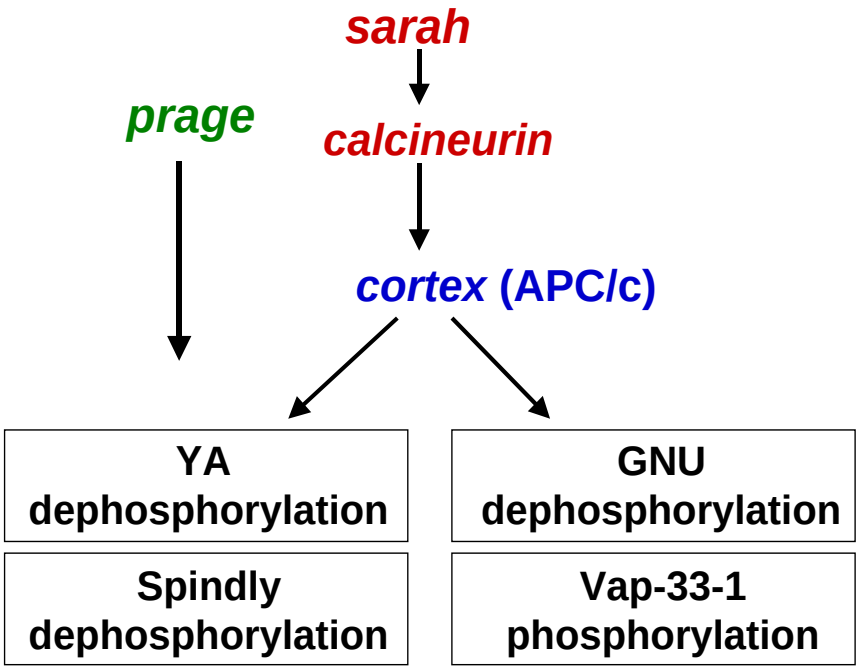


Figure 3.8 Summary of phospho-modulation pathways at egg activation. *Sarah* and calcineurin act in a single pathway with *cortex* to regulate the phosphorylation states of YA, Spindly, GNU and Vap-33-1 at egg activation. *Prage* acts downstream, or in a parallel pathway to regulate only a subset of the proteins that are regulated by *sarah*, calcineurin, and *cortex*.

phosphoproteome of the oocyte, we will gain important insight into how the events of egg activation are coordinated to achieve the oocyte-to-embryo transition.

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CHAPTER FOUR

SCREENING FOR NEW EGG ACTIVATION GENES

4.1 Introduction

Traditional screens for female sterile mutants have identified only a handful of genes that are required for egg activation (reviewed in Chapter One). While female sterility may be a useful filter, defects in a number of other processes (such as oogenesis) can also cause females to be sterile. In addition, genes that are important for egg activation may have pleiotropic effects that make them difficult to identify through traditional female sterile screens. For example, mutations in essential genes would be lethal and could not be studied for roles in egg activation (or fertility) except by analysis with germline clones. The egg activation genes that have been identified so far such as *sra* and calcineurin (Horner et al., 2006; Takeo et al., 2010; Takeo et al., 2006) and other genes reviewed in Chapter One, have provided important insight into how egg activation is regulated. However, without knowing the full suite of genes/proteins that act to allow or regulate egg activation, our understanding of this process will remain incomplete. In this chapter I summarize the results from two alternative genetic screens that aimed to identify new egg activation genes.

In Chapter Two I described a proteomic approach to identify proteins that are phospho-regulated during egg activation. Since these proteins are not only present, but also regulated, in the oocyte/activated egg, we hypothesize that they are an enriched candidate set to test for new players in the oocyte-to-embryo transition.

Chapter Two contains results of knocking down 18 of these candidates by RNAi. Those were the first genes tested and include a promising new egg activation/early development gene, *mrityu*. The present chapter presents the results of testing additional candidates through germline-specific RNAi, P-element insertions, and tissue-specific gene expression. Proteins that are phospho-regulated during egg activation may be important for maintaining the arrested state of the mature oocyte prior to activation, for regulating the events of egg activation, or for early embryogenesis immediately after egg activation. By screening the proteomic candidates for female sterility when the genes encoding these proteins are knocked down in the female germline, we should identify genes/proteins that act at any of these three stages. Further analysis will then allow us to dissect when and how theses genes and proteins function.

Another approach for identifying new factors important for egg activation is to search for genes that interact with known egg activation genes. Therefore, we performed a screen to identify genes that genetically interact with calcineurin in the oocyte. This screen was initiated by the lab of Dr. Toshiro Aigaki, who kindly provided us with flies and preliminary findings. To study the role of calcineurin during female meiosis, the Aigaki lab created transgenic flies that express a constitutively active form of calcineurin (CnA^{act}) in the female germline (Takeo *et al*., 2006). They found that CnA^{act} females are sterile (Takeo *et al*., 2006). The Aigaki lab then performed a deficiency screen to identify regions of the genome that, when hemizygous, suppressed the sterility caused by CnA^{act}. They found 17 different deficiencies that restored fertility to CnA^{act} females (T. Aigaki, personal

Table 4.1 Deficiencies that strongly suppressed the CnA^{act} female sterility.

Listed are the 17 deficiencies that the Aigaki lab found to suppress the sterility caused by expression of CnA^{act} in the female germline, the hatchability for each deficiency, and the number of genes within each deficiency (T. Aigaki, personal communication).

Name of deficiency	Deletion interval	Egg Hatchability(%)	Number of genes within the interval
Df(1)JC70	4C15-5A02	70.6	133
Df(1)v-L15	9B01-10A02	ND	221
Df(2L)BSC5	26B01-26D02	1.3	94
Df(2L)BSC17	30C03-30F01	5.0	87
Df(2L)BSC50	30F04-31B04	16.4	81
Df(2R)ST1*	42B03-43E18	77.0	273
Df(2R)cn9*	42E-44C	ND	336
Df(2R)BSC29	45D03-45F06	1.9	63
Df(2R)stan1	46D07-47F16	29.2	205
Df(2R)Exel7130	50D04-50E04	ND	37
Df(2R)BSC45	54C08-54E07	13.5	102
Df(2R)14H10Y-53	54D01-54E07	45.9	87
Df(2R)Egfr5*	57D02-58D01	28.5	237
Df(2R)X58-12	58D01-59A	19.4	143
Df(2R)Px2	60C05-60D10	12.1	94
Df(3L)AC1	67A02-67D13 or 67A05-67D13	50.6	219 or 214
Df(3R)Antp17	84B01-84D12 or 84A01-84D14	ND	162 or 239

ND: Not determined
*These three deficiencies are being studied by the Aigaki lab.

communication). After selecting specific regions on which to focus their own studies, T. Aigaki sent our lab the CnA^{act} stocks and the list of deficiencies that suppressed sterility. These deficiencies contained anywhere from 37 to 200+ genes (Table 4.1; T. Aigaki, personal communication). Our goal was to identify which gene(s) in each deficiency was responsible for suppressing the CnA^{act} sterility. We focused on the five deficiencies with the highest level of suppression of CnA^{act} sterility (determined by the greatest percentage of progeny compared to number of eggs laid), and either identified the gene or narrowed down the region that interacts with calcineurin.

4.2 Materials and Methods

Flies: To knock down genes by RNAi, we used transgenic flies produced by the Transgenic RNAi Project (TRiP) at Harvard. Lines were either requested directly from the TRiP or were ordered from the Bloomington Stock Center. Specific stock numbers for each gene are included in the tables in the Results section. To achieve knockdown specifically in the female germline, lines containing the RNAi constructs were crossed to either *nanos*-GAL4 (Bloomington stock #4937) or the Maternal Triple Driver (MTD)-GAL4 (Bloomington stock #31777). P-element insertion lines and deficiency lines for the CnA^{act} screen, or to test proteomics candidates, were ordered from the Bloomington Stock Center; the specific stock numbers are in the tables in the Results section. Other stocks used were: *Vap-33-1*$^{\Delta 20}$/FM7;; *UAS-Vap*, *Vap-33-1*$^{\Delta 448}$/FM7;; *UAS-Vap*, D42-GAL4, and C164-GAL4 (kind gifts of H. Bellen; Pennetta *et al*., 2002) and *elav*-GAL4 (Bloomington stock #8760) to test for a role of Vap-33-1 in the female germline, y^1 w^{67c23}; *P{EPgy2}mri*^{EY01340} (Bloomington stock # 15834)

and w^{1118}; *ry Dr P{Δ2-3}99B*/TM6C *Sb* (Bloomington stock #5908) to make *mrityu* mutants through imprecise P-element excision mutagenesis, and w^{1118};; *nanos*-GAL4, *UASp-Pp2B-14D*^{act}/TM3 *Sb Ser* for expression of constitutively active calcineurin in the female germline (kind gift of T. Aigaki; Takeo *et al*., 2006).

Crosses: TRiP lines were crossed to *nanos*-GAL4 or MTD-GAL4 to knockdown genes of interest specifically in the female germline. Control females were *nanos*-GAL4/balancer or MTD-GAL4/balancer siblings from crosses in which the TRiP lines were balanced.

To test for the role of Vap-33-1 in the female germline *Vap-33-1*$^{\Delta 20}$/FM7;; *UAS-Vap* or *Vap-33-1*$^{\Delta 448}$/FM7;; *UAS-Vap* females were crossed to three different driver lines (D42-GAL4, C164-GAL4, and *elav*-GAL4) to express *Vap-33* in the nervous system. Male progeny that did not contain the FM7 balancer chromosome were then crossed back to *Vap-33-1*$^{\Delta 20}$/FM7;; *UAS-Vap* or *Vap-33*-1$^{\Delta 448}$/FM7;; *UAS-Vap* females. Non-balanced female progeny from this cross are *Vap-33-1* nulls expressing *Vap-33* "only" in the nervous system (however, two of these drivers appear to produce expression in the germline, see Results).

For the CnA^{act} suppression screen, virgin females from deficiency or P-element insertion lines were crossed to *nos*>CnA^{act} males. Female progeny carrying both CnA^{act} and the deficiency/mutation of interest were collected by selecting against all balancer chromosomes.

Fertility assay: Virgin females, aged 3-5 days, were individually mated with wild-type (ORP2) males. Following copulation, males were removed and females were allowed to lay eggs for 24 hours. Every 24 hours, for 3-5 days, females were

transferred to a fresh vial and the number of eggs laid in the previous vial was counted. In this way, the total number of eggs laid by each female was determined. The total number of pupae or adult progeny in each vial was also counted to determine the fertility of each female. Hatchability was determined by dividing the total number of adult progeny by the total number of eggs laid. The average hatchability is presented for each genotype. For the CnA^{act} screen, the number of eggs laid was not always counted and results may instead be presented as the average number of pupae/adult progeny over 3 days. N values are presented in Results.

Immunostaining and microscopy: Embryos or ovaries were fixed in methanol/heptane and stained as described in Horner et al. (2006). Guinea pig anti-Vap-33-1 antibody was used at 1:200 (kind gift of H. Bellen; Pennetta *et al*., 2002). Mouse anti-tubulin antibody was used at 1:400 (Sigma, St. Louis, MO, catalog#T5168) and Alexa secondary antibodies were used at 1:200 (Invitrogen, Grand Island, NY). RNaseA (Roche, Indianapolis, IN) was added at a final concentration of 5 μg/ml and propidium iodide (Molecular Probes, Eugene, OR) was used at a final concentration of 10 μg/ml. Images were collected using a Leica TCS SP2 confocal microscope as described in (Horner et al., 2006).

4.3 Results and Discussion

Candidates from phospho-proteomics:

Insertion lines

The ability to knock down genes in the female germline using RNAi is a relatively recent advancement in Drosophila RNAi techniques (Ni *et al*., 2011). Prior

to the availability of these lines, I searched for transposable-element insertions in the genes encoding each of the 311 proteins that we identified in the phospho-proteomics experiments (Chapter Two) to test if the insertions impaired female fertility. I ordered 19 lines from the Bloomington stock center for which the fertility and/or lethality was not described on Flybase, and one female-sterile allele with an unknown arrest point (flybase.org). Of these 20 lines, 9 were balanced and there were no homozygotes within the stock, suggesting that they contain mutations that affect essential genes. I also failed to obtain homozygotes from the one stock that was listed as a female-sterile allele (FBal0008607). To determine if the genes affected by these mutations act during egg activation will require germline clonal analysis. Another six lines were homozygous viable and fertile. Since the mutations in these lines may only partially knockout gene function, or not affect it at all, testing of additional alleles and/or RNAi lines will be important to determine if the genes mutated in these six lines are important for egg activation.

Of the remaining four lines, one (FBal0216980, Smg6) laid fewer eggs than controls and the other three (*CG14749*^{DG30308}, FBal0219308; *ssp3*^{KG05595}, FBal0148274; *CG5245*^{A366}, FBal0222431) had lower hatchability than controls. These results are summarized in Table 4.2. I further investigated one of the lines with low hatchability. This line has an insertion predicted to affect the gene *CG5245*, so I crossed the stock to a deficiency in the same region. Flies that were hemizygous for the *CG5245*^{A366} allele had wild-type hatchability (98% hatch for *CG5245*^{A366}/Df compared to 22% hatch for *CG5245*^{A366}/*CG5245*^{A366}). I confirmed these results by testing a second allele of *CG5245* (*CG5245*^{C316}, FBal0216993) which is also fully

Table 4.2 P-element insertion lines for candidates from phospho-proteomics.

For *CG14749*, n = 10 (sibling control n = 4); *short spindle 3*, n = 8 (sibling control n = 10); and *CG5245*, n = 7 (control n = 7).

Gene	Stock #	Phenotype
CG14749	21314	low hatchability (86.8%, p=0.005)
short spindle 3	14597	low hatchability (89%, p=0.026)
CG5245	16099	low hatchability (39%, p=0.006)
Smg6	16301	low egg count
hook-like (female sterile)	6102	no homozygotes
CG10588	21529	lethal
CG11188	18112	lethal
CG6509	13692	lethal
D1 chromosomal protein	17340	lethal
splicing factor Srp54	20545	lethal
CG11414	24087	lethal
CG12945	17145	lethal
CG31368	17825	lethal
CG5602	18387	lethal
CG10103	27211	fertile
CG1910	21055	fertile
RhoGAP92B	15776	fertile
CG2091	21567	fertile
CG11844	23270	fertile
CG16952	19698	fertile

fertile. Therefore, it appears that there is an additional mutation segregating with the *CG5245*^{A366} allele that affects female fertility independent of *CG5245* gene function.

RNAi knockdown

Once RNAi became an available tool for knocking down genes in the female germline (Ni *et al*., 2011), I began using this method to test candidates from the proteomics experiments for roles in egg activation and early embryogenesis. I knocked down candidate genes specifically in the female germline by driving RNAi with a *nanos*-GAL4 or MTD-GAL4 driver and testing the fertility of these females. Fertility was examined by counting the number of eggs laid and the number of progeny produced from those eggs (either pupae or adult flies) to determine the percent that hatched. Maternal effect genes that play a role in egg activation or embryogenesis should have low hatchability values. I did not test genes with a known role in female fertility, that have already been tested by germline clonal analysis, or have fertile null alleles reported (flybase.org). All other candidates were tested for which an RNAi line was available from the TRiP as of February 2012.

Seventy-nine lines have been tested, corresponding to 71 different genes (Table 4.3; this includes the 18 genes reported in Chapter Two). The egg counts and progeny counts for some of these lines were performed by an undergraduate, Lina Abdul Karim, who worked with me in the summer of 2011. Of the 71 genes tested, 16 produced zero, or very few, mature oocytes, suggesting that these genes are necessary for oogenesis. With the tools that are currently available, the early action of these genes prevents us from testing any role in egg activation since knockdown during oogenesis prevents the formation of mature oocytes. Another five genes (*Pk92B*,

Table 4.3 Summary of RNAi results.

Fertile means that hatchability was 90% or greater.

*Reduced hatchability is due to results from a single day, while the other two days showed wild-type hatchabilty. These genes should be retested.

Gene	Stock #	Driver	Phenotype
Rtf1	HMS00168	nanos-GAL4	no oocytes
CG9609	HMS01000	nanos-GAL4	no oocytes
CG5602	HMS01036	nanos-GAL4	no oocytes
CG7597	HMS00155	nanos-GAL4	no oocytes
CG7597	GL00031	nanos-GAL4	fertile
NAT1	BL32357	nanos-GAL4	no oocytes
mcm3	BL34686	MTD-GAL4	no oocytes
putzig	GL00373	MTD-GAL4	no oocytes
Tango7	BL32879	MTD-GAL4	no oocytes
lolal	GLV21087	MTD-GAL4	no oocytes
Tcp-1eta	BL34931	MTD-GAL4	no oocytes
Uba1	GL00491	nanos-GAL4	no oocytes
CG2774	GL01089	nanos-GAL4	no oocytes
Spc105R	BL35466	MTD-GAL4	no oocytes
Spindly	BL34933	both	no/few oocytes
Uba2	BL35806	MTD-GAL4	no/few oocytes
cdc37	BL34991	MTD-GAL4	few oocytes
Pk92B	GL00238	MTD-GAL4	30%
Pk92B	BL32464	MTD-GAL4	80%
BRWD3	BL33421	nanos-GAL4	28%
modulo	GLV21029	MTD-GAL4	29%
EDTP	HMS01577	MTD-GAL4	50%
FKBP59	BL35612	MTD-GAL4	60%
mrityu	GL00033	both	0%
PyK	GL00099	nanos-GAL4	0%
alpha-adaptin	BL32866	both	0-3%
myopic	BL32916	MTD-GAL4	0%
myopic	HMS00836	nanos-GAL4	fertile
Bre1	GL00368	MTD-GAL4	0%
Nipped-B	GL00574	MTD-GAL4	>1%
Nipped-B	BL32406	MTD-GAL4	14%
14-3-3 epsilon	BL34884	MTD-GAL4	0%
14-3-3 epsilon	BL35441	MTD-GAL4	0%
mod(mdg4)	BL32995	MTD-GAL4	0%
mod(mdg4)	BL33907	MTD-GAL4	3%
Apc	HMS 00188	nanos-GAL4	fertile
RPA2	HMS01061	nanos-GAL4	fertile
Sodh-1	HMS01028	nanos-GAL4	fertile
5-HT1A	HMS00823	nanos-GAL4	fertile
RhoGAP92B	HMS00268	nanos-GAL4	fertile
eif-4A	HMS00927	nanos-GAL4	fertile
Rasputin	HMS00269	nanos-GAL4	fertile

Gene	Stock #	Driver	Phenotype
Hsc/Hsp70-interacting protein related	HMS00988	nanos-GAL4	fertile
Hsc/Hsp70-interacting protein related	BL32900	MTD-GAL4	fertile
Jarid2	BL32891	nanos-GAL4	fertile
CG14446	BL32487	nanos-GAL4	fertile
26-29kD-proteinase	BL32887	nanos-GAL4	fertile
SET domain binding factor	BL32419	nanos-GAL4	fertile
SF2	BL32367	nanos-GAL4	fertile
CG3689	BL32883	both	fertile
XNP	BL32894	nanos-GAL4	fertile
CG13350	BL32968	nanos-GAL4	fertile
Eps-15	HMS00947	nanos-GAL4	fertile
Eps-15	HMS00893	nanos-GAL4	fertile
D1	HMS00061	nanos-GAL4	fertile
Ptp61F	BL32426	nanos-GAL4	fertile
CG18259	BL34081	MTD-GAL4	fertile
Hsp27	BL33007	MTD-GAL4	fertile
CG42669	BL34016	MTD-GAL4	fertile
Hsp26	GL00329	MTD-GAL4	fertile
Cysteine proteinase-1	BL32932	MTD-GAL4	fertile
Hsc70Cb	BL33742	MTD-GAL4	fertile
CG5527	BL34531	MTD-GAL4	fertile
CG9705	GLV21074	MTD-GAL4	fertile
CG8223	GLV21084	MTD-GAL4	fertile
Df31	GLV21083	MTD-GAL4	fertile
ACXD	GL00425	MTD-GAL4	fertile
Aut1	BL34359	MTD-GAL4	fertile
Gadd45	BL35023	MTD-GAL4	fertile
CG10103	BL34979	MTD-GAL4	fertile
RhoGAP68	GL00580	MTD-GAL4	fertile
CG30101	GL00602	MTD-GAL4	fertile
RhoGAP1A	BL33390	MTD-GAL4	fertile
dikar	BL32397	MTD-GAL4	fertile
Med1	BL34662	MTD-GAL4	fertile
CG4968	BL35615	MTD-GAL4	fertile
Gp93	BL34346	MTD-GAL4	fertile
l(3)82Fd	GL00605	MTD-GAL4	65%*
stathmin	GL01099	MTD-GAL4	72%*

BRWD3, *modulo*, *EDTP*, and *FKBP59*) caused reduced hatchability (ranging from 30%-60% hatchability) when knocked down. Since these genes are only knocked down, it is possible that complete sterility may be observed if gene function is removed entirely. Therefore, these five genes are good candidates for further study with null mutations and possibly germline clone analysis. Finally, knocking down eight different genes produced complete (or nearly complete) sterility. One of these genes, *mrityu*, is discussed in Chapter Two and below. The other seven genes are *PyK*, *alpha-adaptin*, *myopic*, *Bre1*, *Nipped-B*, *14-3-3 epsilon*, and *mod(mdg4)*.

I examined the embryos laid by females knocked down for each of these seven genes to determine the period of development that is affected when expression of these genes is reduced. I stained 1-3 hour old embryos with propidium iodide and anti-tubulin antibody, to visualize DNA and microtubules, respectively. When *PyK*, *alpha-adaptin*, *myopic*, and *Nipped-B* are knocked down, embryos are able to undergo multiple rounds of mitosis as seen by the numerous mitotic nuclei present in these eggs (Figure 4.1 and results not shown). This suggests that these genes either function after the early stages of embryogenesis, or that knockdown is not complete enough for us to observe their earlier roles. When *Bre1*, a gene involved in histone modification and Notch signaling (Bray et al., 2005), is knocked down 11/16 embryos appeared to arrest at an early point in embryogenesis while the remaining five embryos had gone through many rounds of mitosis. This could again be due to variability in the level of knockdown achieved by RNAi. Since null alleles of *Bre1* are lethal, further experiments to determine the role of *Bre1* in the mature oocyte and activated egg will likely require germline clone analysis. Knockdown of *mod(mdg4)* in the female

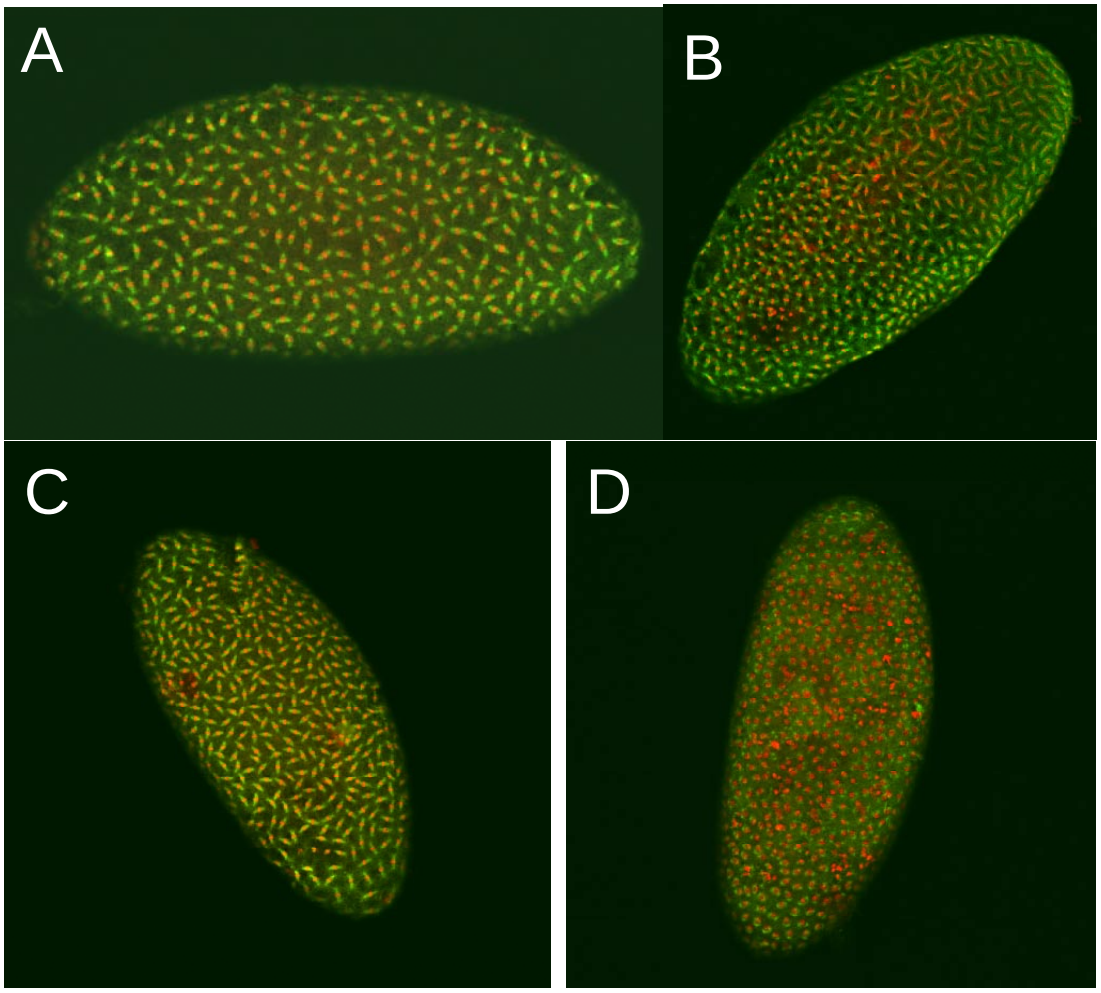


Figure 4.1 Embryos arrest after the early syncytial divisions when *Pyk*, *alpha-adaptin*, or *myopic* are knocked down in the female germline.

1-3 hour embryos laid by (A) control females or females knocked down for (B) *PyK*, (C) *alpha-adaptin*, or (D) *myopic*. Females are sterile when any of these three genes are knocked down in the germline. Embryos were stained with anti-tubulin antibody and propidium iodide to visualize microtubules and DNA, respectively. None of these embryos show an early arrest phenotype. Instead they all have gone through many rounds of embryonic mitosis and look similar to control embryos.

germline also leads to an early arrest, this time in all embryos observed (n=15). Additional experiments are required to determine the exact arrest point of these embryos. *Mod(mdg4)* is involved in chromosome segregation during male meiosis and may play a similar role in female meiosis (flybase.org). Finally, knockdown of *14-3-3 epsilon*, another gene that plays a role in cell cycle regulation (flybase.org), produced embryos with what appeared to be large DNA aggregates. Examining earlier stage embryos, or possibly mature oocytes, may allow us to determine when *14-3-3 epsilon* first acts and how it causes this aggregation later in embryogenesis.

Vap-33-1

Vap-33-1 is one of the proteins that I identified in Chapter Two as changing in phosphorylation state between mature oocytes and activated eggs. In Chapter Three I also showed that this phosphorylation change is dependent on the egg activation genes *sarah* and *cortex*. Next, I was interested in determining what role Vap-33-1 may play during egg activation. Null mutations of *Vap-33-1* are lethal and at present there is no RNAi line to knock down *Vap-33-1* in the female germline (Pennetta *et al*., 2002). Therefore, to test the role of *Vap-33-1* in the female germline, I took advantage of the fact that expression of *Vap-33-1* in the nervous system is sufficient to rescue the lethal phenotype of a *Vap-33-1* null mutation (Pennetta *et al*., 2002).

In theory, expression of *Vap-33-1* solely in neurons, in a *Vap-33-1* null background, should mean that there is no *Vap-33-1* expression in the germline. To express *Vap-33-1* in neurons I used three different nervous system drivers: elav-GAL4, D42-GAL4, and C164-GAL4. When driving *Vap-33-1* expression with either *elav*-GAL4 or D42-GAL4 in a *Vap-33-1* null background, I found that females were

completely fertile. However, Western blots revealed that mature oocytes from these females expressed Vap-33-1 protein at levels comparable to balancer control siblings carrying an endogenous copy of *Vap-33-1* (results not shown). In contrast, when *Vap-33-1* expression was driven by C164-GAL4 in a *Vap-33-1* null background, females did not lay any eggs over the course of seven days. Dissection of these females revealed that they did not produce any mature oocytes. This indicates that *Vap-33-1* expression is required for oogenesis and prevents us from determining its role at egg activation at this time.

In addition to the role and regulation of Vap-33-1, I also briefly examined the localization of Vap-33-1 in the developing oocyte and early embryo. I found that Vap-33-1 is enriched in the developing oocyte and localized to the cytoplasm (Figure 4.2A). In embryos, Vap-33-1 appears to associate with the spindle envelope during mitosis (Figure 4.2B) and may remain peri-nuclear during interphase (Figure 4.2C). In the future, it will be useful to look at Vap-33-1 localization in mature oocytes. Since Vap-33-1 phosphorylation is regulated by *sra* and *cortex*, it will also be of interest to observe Vap-33-1 localization in embryos laid by these mutants.

Mrityu

In Chapter Two I presented results showing that embryos laid by females with reduced levels of *mrityu* in the germline (*mri* RNAi embryos) arrest early in embryogenesis. The majority of these embryos arrested during the first or second embryonic mitosis, and always during metaphase (Chapter Two). Since this arrest is right after egg activation, I examined whether Mrityu function is necessary for the phospho-regulation of other proteins that occurs during egg activation. I was

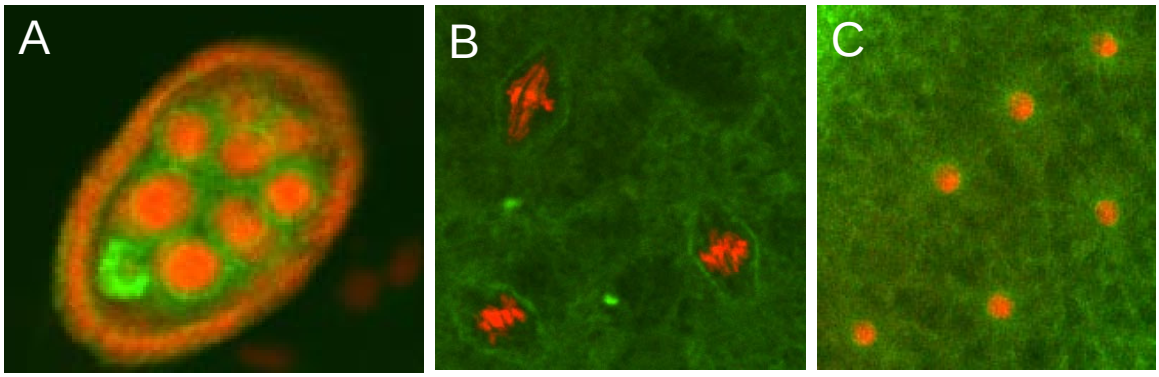


Figure 4.2 Localization of Vap-33-1 in developing oocytes and early embryos. Ovaries and embryos were stained to visualize Vap-33-1 (green) and DNA (red). Vap-33-1 is enriched in the developing oocyte (A). In the early embryo, Vap-33-1 appears to associate with the spindle envelope during mitosis (B) and remain peri-nuclear during interphase (C).

especially interested in whether YA was properly dephosphorylated in *mri* RNAi embryos, since YA is required for the first embryonic mitosis (Lin *et al*., 1991; Yu and Wolfner, 2002; Sackton *et al*., 2009). I found that *mri* is not required for the phospho-regulation of YA, GNU, Spindly, or Vap-33-1 (Figure 4.3).

Mrityu was identified by mass spectrometry as a protein that is phosphorylated upon egg activation (Chapter Two). Since loss of maternal *mrityu* results in an early embryonic arrest, one of the next experiments should be to test whether the phosphorylation of Mrityu is important for its function. This will involve mutating the phosphorylated residues identified by mass spec. and creating transgenic flies that express the mutated protein. If the phosphorylation is required for Mrityu to function at egg activation, then mutating these residues to alanine should result in a loss of function phenotype. Additionally, mutating these residues to phospho-mimetic amino acids could result in premature activation in oocytes. In order to observe the effects of the mutated transgene, we will need to express it in a *mri* null background to remove the effects of the endogenous protein. A null mutant should also allow us to determine a more exact arrest point, as the variability that we see with RNAi knockdown is likely due to the small amount of *mri* expression that still occurs in these oocytes/embryos.

At present, there is no null allele of *mri* reported. Therefore, I tried to create a null allele through imprecise P-element excision mutagenesis. The Bloomington stock center has one line with a P-element insertion in the 5' UTR of *mri*. I crossed this line to a line carrying the Δ2-3 transposase, and then carried out single-pair matings of $mri^{EY01340}$/*Dr Δ2-3* males crossed to w^{1118};; +/TM3 *Sb* virgin females. Excision events were identified by a w^- phenotype, due to the loss of the mini-white gene in the

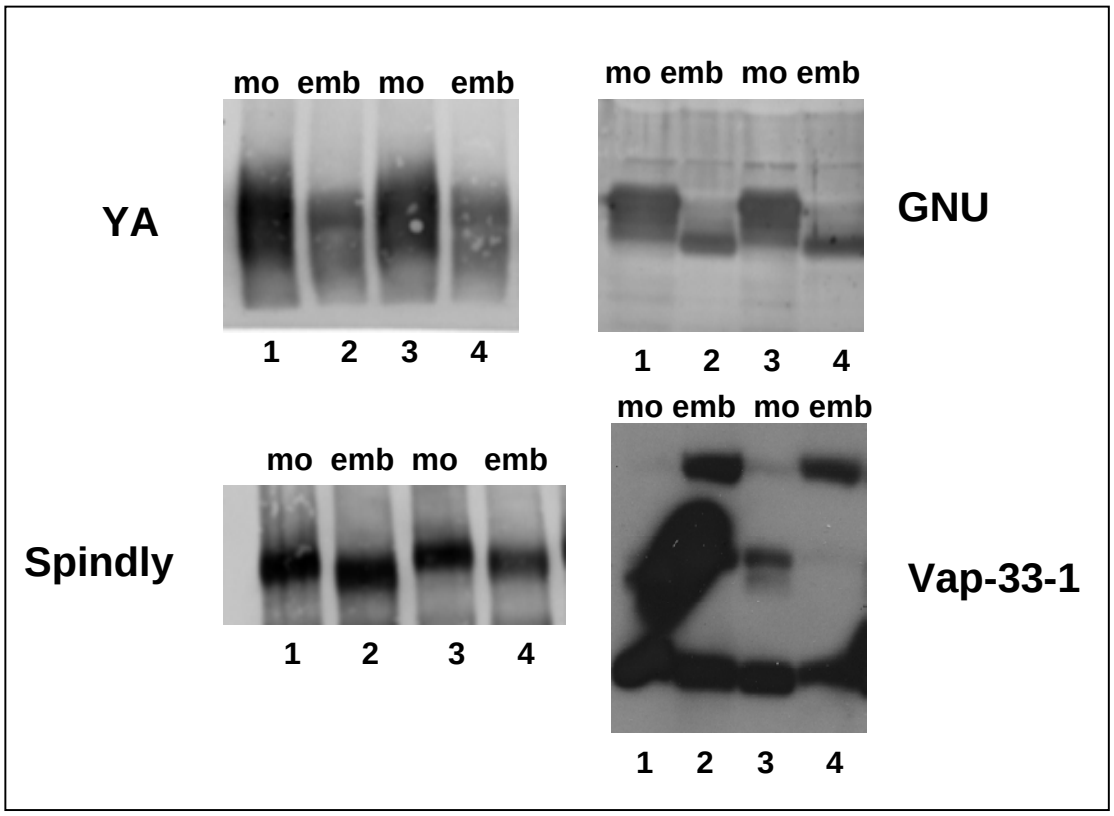


Figure 4.3 Protein phosphorylation changes take place normally in *mri* RNAi embryos. No differences are seen in the phosphorylation states of YA, GNU, Spindly or Vap-33-1 for control and *mri* RNAi mature oocytes (mo; compare lanes 1 and 3) or for control and *mri* RNAi 0-1 hour embryos (emb; compare lanes 2 and 4).

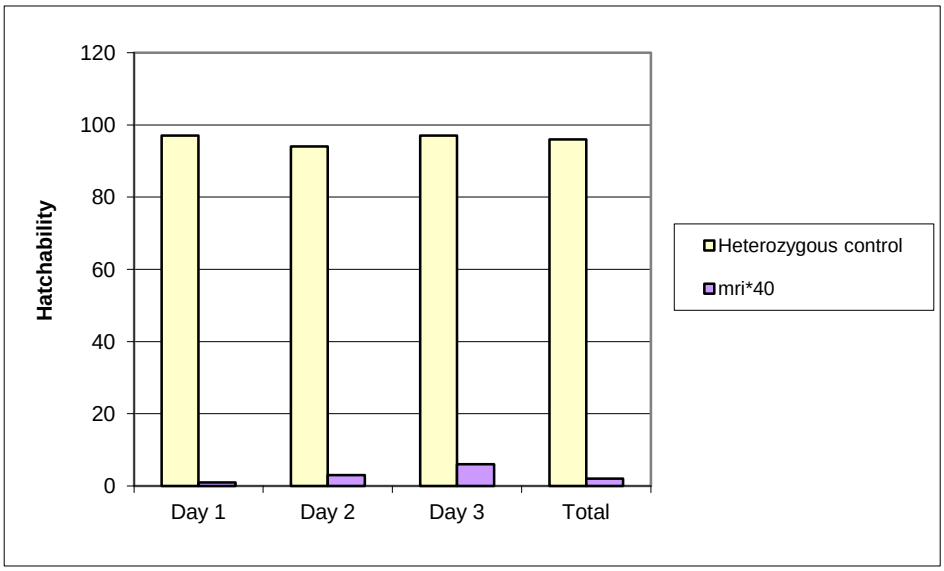
P-element. I collected approximately 60 *w*;; Dr^{+}/TM3 *Sb* progeny. These were then individually mated to *w*;; $mri^{EY01340}$/TM3 *Sb*. The white-eyed, *Stubble* progeny were collected and siblings were mated to establish *mri* mutant lines.

The majority of the excision lines were homozygous viable and fertile. Since RNAi knockdown of *mri* results in a female sterile phenotype, I concluded that these lines were either precise excisions, did not excise the entire P-element, or did not disrupt enough of the surrounding sequence to affect the coding sequence of *mri*. But nine lines were homozygous lethal; progeny always possess the balancer chromosome. This suggested that *mri* may be an essential gene. However, the gene immediately upstream of *mri* is *rhinoceros*, which is known to be essential (Voas and Rebay, 2003). Therefore, it is also possible that the homozygous lethal P-element excision lines are lethal due to disruption of *rhinoceros* rather than *mri*. In support of this idea, I also established a single line (mri^{*40}) that produces some homozygotes and affects female fertility. Only 2% of the eggs laid by mri^{*40}/ mri^{*40} females hatch (Figure 4.4A). These females also laid a reduced number of eggs on the second and third day after mating, compared to heterozygous control females (Figure 4.4B). In the future the excision lines will need to be sequenced to determine the nature of the mri^{*40} and other alleles. Complementation test with *rhinoceros* mutants will help to establish whether lethality of the other lines is in fact due to disruption of *rhinoceros*.

Suppressors of the sterility caused by expressing constitutively active calcineurin in the female germline:

We examined five different deficiencies that were previously found to suppress the sterility caused by expressing CnA^{act} in the female germline. These deficiencies

A



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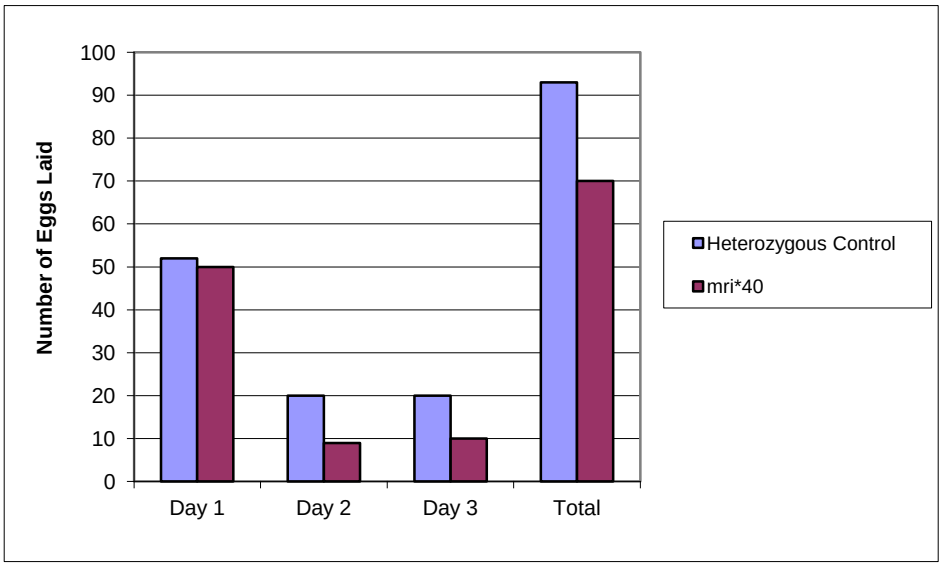

Figure 4.4 Hatchability and number of eggs laid for mri^{*40} homozygotes and heterozygous controls.

Mri^{*40} homozygotes are almost completely sterile for the first three days after mating (A). In addition, *mri*^{*40} homozygous females lay the same number of eggs as controls on day 1 but lay reduced numbers of eggs on days 2 and 3 (B). Bars show the average hatchability/ number of eggs laid (n = 13 for *mri*^{*40} homozygotes, n = 14 for controls).

are Df(1)JC70, Df(2R)14H10Y-53, Df(2R)X58-12, Df(2R)stan1, and Df(3L)AC1. We picked these deficiencies because they produced some of the greatest levels of suppression, as determined by the percentage of laid eggs that hatched.

The majority of this project was carried out by a talented undergraduate student, Rebecca Zuckerman, whom I mentored for two and a half years. Her results are written up in her senior honors thesis (R. Zuckerman, 2011) and are summarized here in Table 4.4. Rebecca identified one P-element insertion within the region of the genome deleted by Df(1)JC70 that restored fertility to wild-type levels. When females carried one copy of P{GT1}BG01861 and the CnA^{act} transgene, 92.4% of the laid eggs hatched. P{GT1}BG01861 is located just downstream of *CG6927* and just upstream of another gene, *CG32772*. Since another P-element insertion directly in the coding region of *CG32772* does not restore fertility to CnA^{act} females, we concluded that *CG6927* is the gene responsible for the observed suppression by P{GT1}BG01861. The function of *CG6927* is unknown, though it is predicted to function in ion transport based on domain structure (flybase.org).

One experiment that we tried, but did not complete, was to test if this same P-element insertion can also suppress *sra* mutations. But unlike the CnA^{act} females, *sra* is only sterile when homozygous or hemizygous and required a more extensive crossing scheme to get females deficient in *sra* and carrying the P-element insertion.

Table 4.4 Summary of results of CnA^{act} suppressor screen from the thesis of R. Zuckerman (2011).

Deficiency	Gene/Deficiency	n	Hatchability	P-value
Df(1)JC70	*CG3009*	9	0.108	0.086
	rap	9	0.005	0.006*
	Pp2C1	7	0.067	0.182
	ctp	10	0.001	0.002*
	CG7010	7	0.124	0.135
	CG7024	10	0.001	0.018*
	CG6986	8	0.000	0.002*
	CG2861	10	0.000	0.001*
	CG6927	**23**	**0.924**	**<0.0001****
	CG32772	10	0.048	0.612
	CG42594	10	0.005	0.219
	CG6903	8	0.044	0.627
	CG4068	12	0.118	0.883
	Ptp4E	6	0.013	0.128
	CG32767	8	0.018	0.193
	ovo	9	0.038	0.257
	rg	7	0.000	0.004*
	cdk7	3	0.005	0.130
	CanB	8	0.079	0.154
	sans fille	17	0.104	0.366
	CG4198	10	0.032	0.336
	XRCC1	8	0.016	0.230
	CG15930	10	0.003	0.003*
	sk	10	0.000	0.002*
	NAAT1	9	0.000	0.001*
	yu	6	0.059	0.581
	CG15784	10	0.053	0.709
	CG4165	10	0.001	0.037*
	CG12730	15	0.093	0.695
	Vsx2	7	0.000	0.007*
Df(2R)14H10Y-53	***Df(2R)Exel7149***	**8**	**0.644**	**0.0002****
	Df(2R)BSC347	**9**	**0.524**	**0.0012****
	Df(2R)BSC348	10	0.024	0.4474
	CG10936	8	0.079	0.678

Deficiency	Gene/Deficiency	n	Hatchability	P-value
Df(2R)14H10Y-53	*rhi*	7	0.025	0.283
	CG18186	8	0.000	0.003*
	CG30106	9	0.037	0.351
	eIF3-S8	**10**	**0.187**	**0.007****
	CG30108	8	0.042	1.000
	CG30109	8	0.081	0.116
	CG6459	6	0.049	0.707
	sema-1B	10	0.005	0.098
	HPS4	10	0.061	0.367
	swi2	10	0.093	0.345
Df(2R)X58-12	*Df(2R)BSC597*	9	0.024	0.1046
	Df(2R)Exel7171	10	0.036	0.8237
	Df(2R)Exel7173	**10**	**0.145**	**0.009****
	Df(2R)BSC598	**10**	**0.182**	**0.0104****
	CG3927	12	0.022	0.086
	CG34445	10	0.091	0.384
	RpS24	**3**	**0.630**	**<0.0001****
	Ugt58Fa	9	0.021	0.134
	CG2852	9	0.119	0.490
	CG13510	9	0.013	0.044*
	CG42565	**11**	**0.630**	**<0.0001****
	CG42566	10	0.005	0.006*
	CG3746	11	0.006	0.008*
Df(3L)AC1	*Df(3L)BSC390*	10	0.002	0.0127*
	Df(3L)BSC393	8	0.002	0.0148*
	Df(3L)BSC576	10	0.002	0.0076*
	Df(3L)BSC283	9	0.094	0.4474
Df(2R)stan1	*Df(2R)BSC281*	10	0.074	0.5281
	Df(2R)ED2098	6	0	0.0223*
	Df(2R)BSC336	9	0.028	0.5694
	Df(2R)BSC231	**10**	**0.14**	**0.0196****

Since *sra* regulates calcineurin (Takeo et al., 2010), there is a strong possibility that genes that interact genetically with calcineurin may also interact with *sra*. In the future it would be useful to test whether any of the genes identified through this screen can also suppress the *sra* phenotype.

Another gene that is within the Df(1)JC70 deficiency that Rebecca tested is *CanB,* which encodes a subunit of calcineurin. Rebecca found that *CanB* did not suppress the sterility caused by CnA^{act}. This result is consistent with the report that *CanB2* is the regulatory subunit present in oocytes, and that *CanB* is not expressed in ovaries (Takeo *et al*., 2010).

For the other four deficiencies that we looked at, Rebecca first narrowed down the possible suppressor genes by testing smaller deletions within each of the four regions. For Df(2R)14H10Y-53, she then found one gene, *eIF3-S8,* that significantly restored fertility to CnA^{act} females (18.7% hatchability compared to 45.9% for the deficiency). For Df(2R)X58-12, Rebecca found that the gene, *CG42565*, restored fertility to CnA^{act} females (63% hatchability). She found a second gene, *RpS24*, which may also be a suppressor, but needs to be retested to confirm the result due to a low number of females (n=3) tested in the first experiment. *EIF3-S8* is a predicted translation initiation factor and *RpS24* encodes a ribosomal protein (flybase.org). Since new protein translation is an event triggered by egg activation, these two genes could play a role in regulating this process. There is currently nothing known or predicted about the function of *CG42565* (flybase.org).

For the final two deficiencies, Df(2R)stan1 and Df(3L)AC1, Rebecca was able to narrow down the region containing the gene(s) that suppressed the CnA^{act} sterility

by showing that seven smaller deficiencies did not contain any suppressors. I then tested 25 genes that were within these smaller areas under the deficiency; 11 within Df(2R)stan1 and 14 within Df(3L)AC1. I also tested two P-element excision lines that Rebecca created to try to knock out *CG6927* (*CG6927*^{5} and *CG6927*^{7}). These results are summarized in Table 4.5. I only counted the number of pupae or progeny produced and did not determine hatchability for these 25 genes. However it allowed me to rule out certain genes and highlights which genes should be retested with both egg counts and progeny counts. In addition there are seven more stocks that still need to be tested: Bloomington stock numbers 6402, 19321, 12683, 16329, 28422, 22448, and 14968, corresponding to genes *tou*, *CG9003*, *Hsp67Bb*, *Hsp67Ba*, *CG3689*, *CG43325*, and *RYBP* respectively. There are also nine other deficiencies for which we have not yet begun to identify the genes that are responsible for the suppression of CnA^{act} sterility. Once we know the identities of these genes we can start to form hypotheses about how they interact with calcineurin and the role they may be playing during meiosis and egg activation.

4.4 Summary

This chapter contains the results from two different screens that have identified new candidates to study for roles in egg activation. Genes such as *mod(mdg4)* and *CG6927* are prime candidates for future studies. I also described experiments directed at understanding the role of Mrityu, and the importance of phosphorylation for its function. In addition, neither screen is completed, suggesting that there are a number of new egg activation/early embryogenesis genes that remain to be discovered by

Table 4.5 Progeny counts from additional genes under Df(2R)stan1 and Df(3L)AC1, testing for suppression of CnA^{act} sterility. In cases where a gene is listed twice, each line represents an independent experiment.

Deficiency	Gene	Stock #	**Avg # Progeny**	**n**
	CG6927^{5}	NA	7	10
	CG6927^{7}	NA	7	10
	CnA^{act}	NA	5	10
	CnA^{act}	NA	2	6
Df(2R)stan1	*CG7759*	18535	7	9
	CG7759	18535	23	4
	Drip	17740	27	5
	CG7777	14750	8	10
	qvr	16588	12	5
	E(Pc)	9396	12	8
	E(Pc)	9396	18	3
	inv	26891	10	5
	en	5343	8	4
	Egm	11133	22	8
	Egm	11133	55	4
	CG9005	12768	4	5
	Roc2	17251	0	5
	Tret1-1	21925	4	4
Df(3L)AC1	*Hsp22*	20055	1	10
	Hsp22	20055	11	5
	Hsp26	20186	6	5
	Hsp23	12545	29	10
	Hsp23	12542	107	4
	Hsp27	20107	3	4
	CG4080	15068	12	5
	eIF-4E	2470	32	8
	eIF-4E	2470	69	5
	Cpr67B	17771	17	10
	Cpr67B	17771	24	5
	CG4022	22477	12	5
	CG3967	17568	11	10

Table 4.5 (continued)

Deficiency	Gene	Stock #	Avg # Progeny	N
Df(3L)AC1	*CG3967*	17568	38	5
	aay	28577	1	4
	shc	14051	9	5
	RpS17	6177	7	8
	MTF-1	9241	9	10
	Nf-YA	14501	8	5

these methods. Knockdown has not been confirmed for any of the genes tested by germline-specific RNAi, so it is possible that some of the genes did not give a phenotype due to insufficient knockdown. In these cases, the genes will need to be tested with additional RNAi lines or by alternative genetic methods. Once we know the players, the next step will be to understand how they work together and how they are regulated to coordinate the various events of egg activation.

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CHAPTER FIVE

DISCUSSION

Egg activation is required for a mature oocyte to become developmentally competent. Prior to egg activation, mature oocytes are held in a poised, but arrested state: paused in a species-specific stage of the cell cycle and stocked full of inactive mRNAs and proteins. Upon an appropriate trigger, such as fertilization, an initiating signal sets off the various events of egg activation. In most species studied to date, this initiating signal appears to be a rise in cytosolic Ca^{2+} levels. Two molecules that transduce the Ca^{2+} signal to regulate egg activation are the kinase CaMKII and the phosphatase calcineurin (reviewed in Chapter One). However, the pathways that connect Ca^{2+} and these upstream effectors to the individual events of egg activation remain largely unknown.

Since CaMKII and calcineurin are both regulators of protein phosphorylation, it is reasonable to predict that one way in which the Ca^{2+} signal is connected to each of the events of egg activation is through changes in protein phosphorylation state. Phosphorylation can rapidly alter a large number of proteins and can have a variety of regulatory effects. I hypothesize that global phosphorylation changes are a key contributor to the complete change in cellular state that a mature oocyte undergoes at egg activation. Drosophila makes an excellent model to study this hypothesis due to the abundance of genetic and molecular tools available in this system. Drosophila also has the advantage that, as in other insects, its egg activation is triggered independent

of fertilization (unlike many other organisms, such as vertebrates and most marine invertebrates). This allows me to specifically study changes and mechanisms associated with egg activation, without the confounding events of fertilization and embryogenesis.

The studies presented in Chapter Two of this dissertation show that large-scale phosphorylation changes take place during egg activation in Drosophila. The proteomic experiments that I performed identified 311 proteins that differ in phosphorylation state between mature oocytes and activated eggs. These experiments inform us about egg activation in multiple ways. First, they show that phospho-regulation does affect a large number of proteins in the mature oocyte. This is consistent with our hypothesis that global phosphorylation changes are a critical aspect of the oocyte-to-embryo transition. As our results are parallel to observations made in sea urchins (Roux *et al*., 2006; 2008), it is likely that dynamic phosphorylation changes are a conserved feature of egg activation.

Second, the identification of hundreds of proteins that are phospho-regulated during egg activation inform us of the biological classes that are important during or immediately after this transition. For instance, I identified proteins involved in protein translation, components of the sumoylation pathway, and zinc-binding proteins. In addition, we can now test each of these proteins individually for roles in egg activation and embryogenesis. So far my mentees and I have tested 71 genes that encode proteins identified by the proteomics experiments (Chapters Two and Four). Thusfar I identified one new gene, *mrityu*, that acts immediately after egg activation during the first few embryonic mitoses. Other promising candidates include *Bre1*, *mod(mdg4)*,

CG6927, and several others, which require further studies to determine their time of action.

Finally, using the phosphorylation states of specific proteins as molecular markers has informed us about how some of these phosphorylation changes are regulated, and the relationship between the egg activation genes *sarah*, *cortex*, and *prage*. In Chapter Three, I showed that *sarah*, *cortex*, and *prage* are all upstream of Spindly dephosphorylation, while only *sarah* and *cortex* are upstream of Vap-33-1 phosphorylation. My data have led to our current model in which *sarah* acts upstream of *cortex* in a single pathway and *prage* acts downstream of, or in parallel to, this pathway.

My studies indicate the importance of understanding the role of phosphorylation in the regulation of egg activation. I have contributed to the identification of new candidates genes that may act at this time and have helped to place some of the previously known genes/proteins, and a couple of new ones, into a pathway. However, a number of important questions still remain. I believe that finding the direct targets of calcineurin (and/or CaMKII in other organisms) will be critical to our understanding of how these proteins direct egg activation. Are these proteins directly responsible for the majority of the phosphorylation changes that take place during egg activation? Or do they only have a small number of direct targets, after which additional phosphorylation changes are regulated by other downstream kinases and phosphatases?

One way to begin asking these questions could be additional proteomic experiments comparing the phosphoproteomes of eggs laid by *sarah* and *cortex*

mutants to the phosphoproteome of wild-type unfertilized eggs. So far we have not seen a difference in the phospho-regulation phenotypes of *sarah* or *cortex*, except in the severity of their effects. Proteomic experiments would allow us to determine to what extent their targets overlap. In addition, proteins that are phosphorylated in *sarah* embryos, but are dephosphorylated in *cortex* and wild-type embryos are good candidates for direct targets of calcineurin.

Another important area of study is to understand the functional importance of these phosphorylation changes. How do the different phosphorylation states of these proteins affect their activity, localization, etc.? One advantage of using IMAC to identify phospho-modulated proteins is that this method identifies specific residues that are phosphorylated. Therefore, if a protein has an interesting loss-of-function phenotype we already have a specific site (or specific sites) for mutational studies. It seems clear that phosphorylation is important. However, a complete understanding of its regulatory role will only be possible when we know not only the global phosphorylation changes, but also how phospho-modification affects individual proteins.

APPENDIX A

INVESTIGATING A ROLE FOR NPLP3 IN THE FEMALE POST-MATING RESPONSE

A.1 Introduction

Mating induces a number of behavioral and physiological changes in the female of Drosophila melanogaster. These changes include increased ovulation and egg laying, changes in feeding behavior, and decreased receptivity to remating (reviewed in Avila *et al*., 2011). Seminal fluid proteins, transferred from the male to the female during mating, are largely responsible for inducing these changes in the female (reviewed in Avila *et al*., 2011). Studies of individual seminal fluid proteins have provided insight into how these proteins function (for examples see LaFlamme *et al*., 2012; Avila and Wolfner, 2009). However, the female molecules that interact with seminal fluid proteins and are required for the post-mating response remain largely unknown.

To identify female genes that may be involved in responding to seminal fluid proteins McGraw *et al*. (2004; 2008) performed microarray studies to investigate gene expression in mated female Drosophila. These studies identified over 1500 genes whose expression changed as a result of mating; 160 of these changes resulted specifically from the receipt of accessory gland proteins (Acps; a subset of seminal fluid proteins) (McGraw *et al*., 2004). To further dissect the influences of specific Acps, additional microarrays were performed with females mated to males lacking one

of four individual Acps (ovulin, Acp36DE, Acp29AB, and Acp62F) (McGraw *et al*., 2008). A large number of gene expression changes were identified by this study, but most of these changes were very small; only 19 genes had a 2-fold or greater change in expression level (McGraw *et al*., 2008). One of the genes that exhibited the largest fold change in expression level was *neuropeptide-like precursor 3* (*nplp3*),. *Nplp3* was expressed at higher levels when females received either Acp29AB (5-fold higher) or Acp62F (2.5-fold higher), as compared to females that did not receive one of these Acps (McGraw *et al*., 2008). These data, along with the prediction that *nplp3* may be involved in neuropeptide hormone activity and signaling (flybase.org; Baggerman *et al*., 2002), led us to focus on this gene for a role in the female post-mating response. Therefore, for my rotation project I used RNAi and a P-element insertion line to examine if expression of *nplp3* affects female fecundity.

A.2 Materials and Methods

Flies: Drosophila melanogaster stocks were raised on yeast-glucose-agar medium at 23 ± 2^{o}C in a 12-h L:12-h D photoperiod. To make *nplp3* "knockouts", a P-element insertion line (Bloomington stock #17988) was crossed to the deficiency line Df(3L)st-f13, Ki rn^{roe-1} p/TM6B, Tb (Bloomington Stock #2993). To knock down *nplp3* by RNAi in the whole female, virgin females carrying an RNAi construct for *nplp3* [from the Vienna Drosophila RNAi Center (VDRC); transformant ID 12972] were crossed to tubulin-GAL4 males. Females knocked down for *nplp3* were selected as Sb^{+}. Balancer siblings were used as controls.

Fecundity assay: Virgin RNAi (or P-element insertion) females and control females, aged 3-5 days, were individually mated with wild-type (Canton-S) males. Following copulation, males were removed and females were allowed to lay eggs for 24 hours. Every 24 hours, females were transferred to a fresh vial and the number of eggs laid in the previous vial was counted. In this way, the total number of eggs laid by each female over 10 days was determined. After 15 days, the total number of adult progeny in each vial was also counted to determine the fertility of each female. Hatchability was determined by dividing the total number of adult progeny by the total number of eggs laid and is presented as the average (± standard deviation) for each genotype. N values are presented in the Results.

RT-PCR: RNA was extracted from whole *nplp3* RNAi or control females. 750 ng of RNA was treated with 1 µl RQ1 DNase (Promega) for 37^{o}C for 30 min. The reaction was stopped with 1 µl RQ1 DNase Stop Solution and a 10 min incubation at 65^{o}C. cDNA was synthesized using Superscript II Reverse Transcriptase (Invitrogen) and according to manufacturer's protocol. cDNA from *nplp3* RNAi and control females was used as the template in a PCR with primers to *nplp3* or the control gene *RpL32* (which is expect to be present in equal abundance in both samples). The reaction was run for 30 cycles. Products were run along with a 1 Kb Plus DNA ladder (Invitrogen) on a 1% agarose gel containing 0.5 µg/ml ethidium bromide.

A.3 Results

I tested whether *nplp3* expression was knocked down when the VDRC *nplp3* RNAi construct (ID# 4873) was driven by tubulin-GAL4. RT-PCR comparing *nplp3*

RNAi females to control females shows that *nplp3* in the RNAi females is expressed at less than 10% of the levels observed in controls (Figure A.1). As a preliminary test to determine if *nplp3* is required for the female post-mating response, I counted the number of eggs laid by RNAi and control females for 10 days. I showed that, in three independent trials, females knocked down for *nplp3* laid significantly less eggs than controls over a 10 day period (Figure A.2). Only one of these trials (trial 2) showed a significant effect on day 1. In trial 2 *nplp3* RNAi females laid significantly more eggs on day 1 than control females ($p = 0.0136$).

I also tested a P-element insertion line as an independent method to remove *nplp3* function. I crossed this line to a deficiency to obtain *nplp3* hemizygous females that carried the P-element. In contrast to knocking down *nplp3* by RNAi, I found no significant difference in the number of eggs laid over 10 days by females hemizygous for the P-element insertion and heterozygous controls (females carrying one copy of the P-element insertion and one wild-type copy of *nplp3*) (Figure A.3). I did see a significant difference in the number of eggs laid on day 1. However, this also contrasted with the RNAi results because the P-element insertion females laid fewer eggs on day 1 than controls ($p = 0.0182$).

A.4 Discussion

The finding that females knocked down for *nplp3* lay fewer eggs after mating than controls provides promising preliminary evidence that *nplp3* expression in the female may contribute to the regulation of the post-mating response. However, many experiments remain to conclusively define a role for *nplp3*. While the VDRC line that

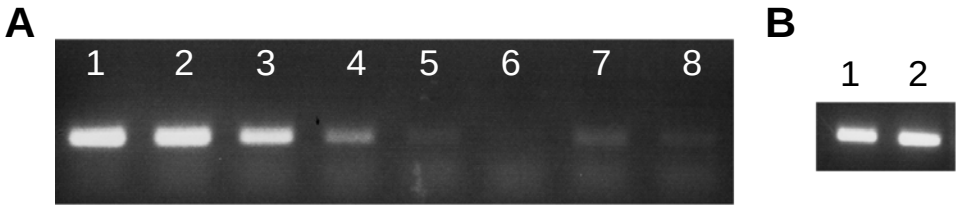

Figure A.1 *nplp3* is knocked down by RNAi. (A) PCR products amplified using *nplp3* primers. Lanes: 1) 100% control cDNA 2) 50% control cDNA 3) 25% control cDNA 4) 10% control cDNA 5) 2.5% control cDNA 6) 1% control cDNA 7) 100% RNAi cDNA 8) 50% RNAi cDNA. (B) PCR products amplified using RpL32 primers. Lanes: 1) 100% control cDNA 2) 100% RNAi cDNA.

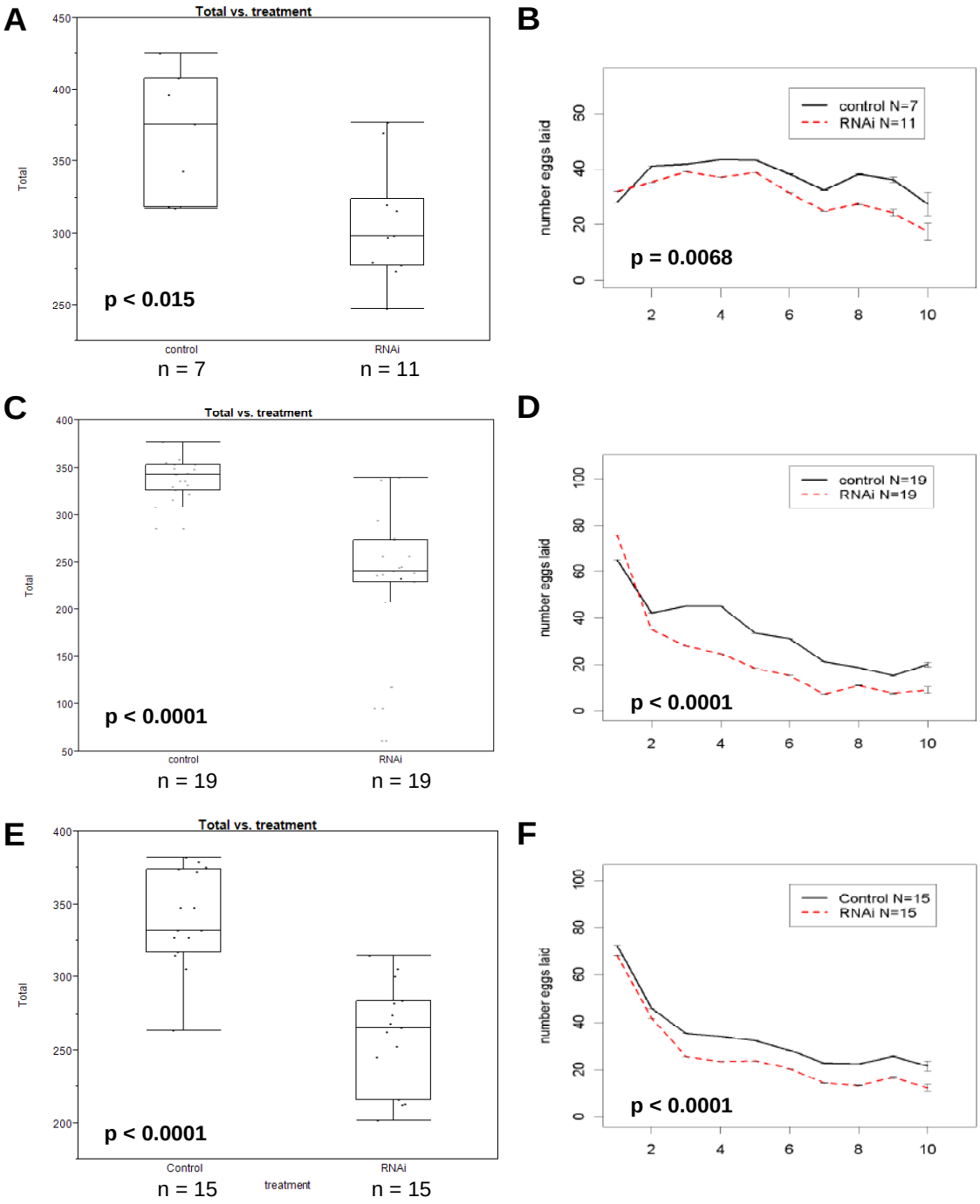

Figure A.2 Females lay significantly fewer eggs when *nplp3* is knocked down by RNAi. Total number of eggs laid in 10 days (A, C, and E) and number of eggs laid each day over 10 days (B, D, and F) for 3 independent trials.

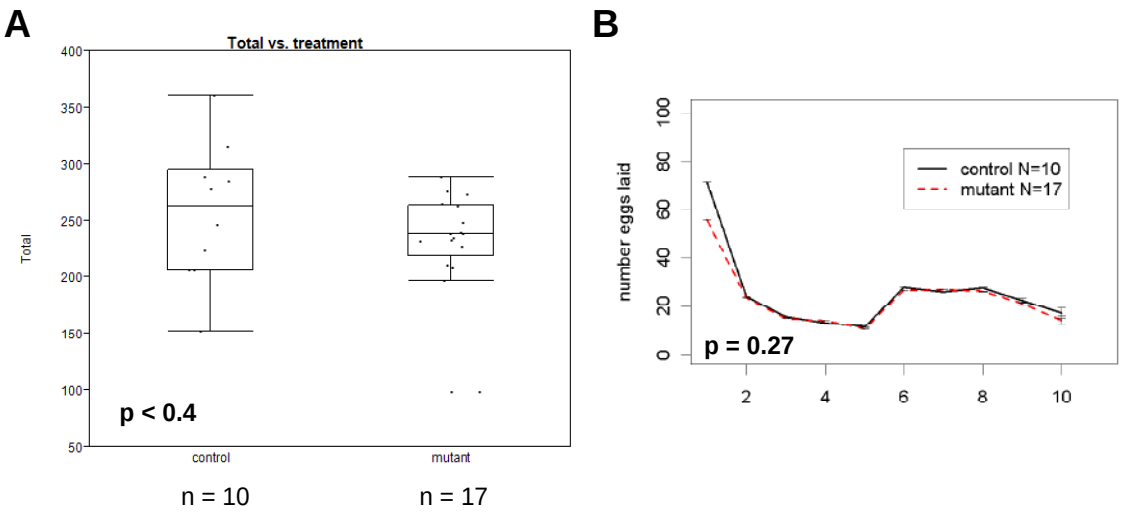

Figure A.3 There are no significant differences in number of eggs laid between hemizygous females with a P-element insertion in *nplp3* and heterozygous controls. Total number of eggs laid in 10 days (A) and number of eggs laid each day for 10 days (B).

I tested successfully knocks down *nplp3*, it unfortunately also has 30 predicted off-targets (stockcenter.vdrc.at). Therefore it is possible that the egg laying phenotype observed for the RNAi females is due to knockdown of one (or more) of these off-targets rather than the knockdown of *nplp3*. An undergraduate in the lab has recently tried to repeat the egg laying results using an independent RNAi line (Bloomington stock #28760). Unfortunately, she found that this line fails to knock down *nplp3* and therefore cannot provide us with the confirmation that we had hoped for (S. Mattei and J. Sitnik, personal communication). There is also a second *nplp3* RNAi line that has become available at the VDRC since I originally performed these experiments (transformant ID #105584). This line has no predicted off-targets and can be tested in the future to confirm that *nplp3* is the gene responsible for the phenotype that I observed.

The fact that the P-element insertion line did not recapitulate the phenotype observed for the RNAi line, could suggest that the phenotype is in fact due to off-target effects. However, the P-element insertion is located in the intron of *nplp3* (flybase.org). Therefore, the P-element may not affect gene function or expression and the level of *nplp3* transcript expression should be determined in these females. If the P-element does not affect gene expression, one possibility for future studies is to use imprecise P-element excision to try to knockout *nplp3*.

Provided we can confirm that *nplp3* affects the number of eggs laid after mating, additional post-mating behaviors should also be tested. In the microarray experiments, *nplp3* expression was affected by Acp29AB and Acp62F (McGraw *et al*., 2008). Acp29AB plays a role in sperm storage in the female (Wong *et al*., 2008)

and is associated with a male's success at sperm competition (Clark *et al*., 1995; Fiumera *et al*., 2005). The localization of Acp62F to the sperm storage organs, suggests that it too may play a role in sperm storage (Lung *et al*., 2002). Gene association studies also show that neuronal genes are enriched among female genes predicted to play a role in sperm competition (Chow *et al*., unpublished data). Taken together, these data suggest that *nplp3* should be tested for a role in sperm competition and/or sperm storage. Further investigations of *nplp3* may help us to understand how the female post-mating response is regulated though female genes that work with transferred seminal fluid proteins.

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